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Functional characterization of Arabidopsis sorting nexin AtSNX2b

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DEDICATION

This Dissertation is dedicated to my parents, Le Ngoc Tuyet and Phan Quang Vinh. Without their support and guidance I would not be here today.

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ABSTRACT

Sorting nexins are conserved proteins that function in vesicular trafficking and are defined by the presence of a phox (PX) domain. Here we present the characterization of *Arabidopsis thaliana* sorting nexin AtSNX2b. The ubiquitously-expressed sorting nexin is a peripherally-associated membrane protein whose PX domain binds to phosphatidylinositol 3-phosphate *in vitro*. AtSNX2b localizes to the *trans*-Golgi network (TGN), prevacuolar compartment and endosomes. Overexpression of GFP-tagged AtSNX2b produces enlarged GFP-labeled compartments that can be also labeled by the endocytic tracer FM4-64, while untagged AtSNX2b overexpression generates compartments which are not as large. Endocytic trafficking of FM4-64 is arrested in cells overexpressing AtSNX2b or GFP-AtSNX2b, while overexpression of AtSNX2b interferes with the transport to the vacuole of proteins containing an N-terminal vacuolar sorting signal but does not affect proteins containing C-terminal vacuolar sorting signal. The intracellular function of AtSNX2b is hypothesized to be in vesicular trafficking from endosomes to the vacuole.

Over-expression and null mutant plants of AtSNX2b show enhanced or reduced root growth, respectively, under nutrient stress conditions during early development of seedlings. *Atsnx2b* knockout (KO) mutant root growth is slowed on nitrogen-deficient, carbon-deficient and high sucrose stress media. Because the *Atsnx2b* KO can be affected by both carbon-deficiency and carbon abundance, it is possible that the AtSNX2b protein contributes to or regulates root growth in developing plants.

To further understand the function of AtSNX2b in vesicular or endosomal trafficking, potential AtSNX2b-interacting factors were examined. Sorting nexins can form homo- and hetero-dimers with other sorting nexins. AtSNX2b colocalizes with AtSNX1 on endosomal

structures, suggesting a potential AtSNX1-AtSNX2b dimer. The analysis of other endosomal localized proteins demonstrated localization of AtSNX2b to BRI1-labeled endosomal structures and this association was supported by detection of AtSNX2b in BRI1-GFP immunoprecipitations. Evidence for TGN function of AtSNX2b was provided by the detection of AtSNX2b in immunoprecipitates of the TGN t-SNARE T7-SYP42. Finally, the analysis of AtSNX2b-interacting factors by co-immunoprecipitation revealed three potential associated proteins that are not predicted to be trafficking components. These proteins are potential cargoes for the AtSNX2b complex and further investigation will determine their function.

CHAPTER 1. INTRODUCTION

Overview

Compartmentalization in eukaryotic cells has allowed complex organisms to increase the efficiency of cellular functions and organization. These cells as a collective contribute to the growth and development of the whole organism. The high degree of organization within cells necessitates precise mechanisms of protein sorting and targeting from their initial site of synthesis to their final destination, and for protein recycling and degradation. Optimum growth requires that the cell be efficiently maintained. Proper maintenance of intracellular components and organelles depends on proper trafficking of macromolecules and membrane targeting by a complex trafficking system (Carter et al., 2004).

In the endomembrane system, protein trafficking involves the processes of synthesis, modification, distribution and internalization of proteins. The endomembrane system is composed of the endoplasmic reticulum (ER), Golgi bodies, prevacuolar compartment (PVC), plasma membrane (PM) and vacuole (Nebenfuhr, 2002). Several membrane recognition systems exist in the cell. One such system involves the distinct subcellular distribution of phosphoinositides on organellar membranes, which allows trafficking components to recognize membranes, and target or trigger signaling processes (Roth, 2004; Takenawa and Itoh, 2006; Krauss and Haucke, 2007; Sasaki et al., 2007). Phosphoinositide-binding proteins play a key role in trafficking and signaling at the membrane by binding and recruiting effector proteins. Phosphoinositide-binding proteins preferentially bind one or more different types of phosphoinositide and can regulate the interaction between proteins and phosphoinositides (Roth, 2004). Sorting nexins are one protein family with a phosphoinositide-binding domain, the PX domain. This protein family has been associated

with several trafficking complexes in yeast and mammals. The overall focus of this research is to study the function of an Arabidopsis sorting nexin. This introduction provides an overview of protein trafficking and discusses specific roles of sorting nexins in protein trafficking.

Trafficking Pathways

Functional compartmentation allows protein production and regulation in the eukaryotic cell. Likened to a production and assembly line, the cell factory necessitates the exchange of proteins, lipids and polysaccharides among a range of membrane compartments. Organelles involved in biosynthetic trafficking include the ER, Golgi and *trans*-Golgi network. The ER is the entry point into the endomembrane system for newly synthesized proteins. Proteins that are destined to go into the ER have a signal peptide which targets these proteins to the ER membrane upon initial synthesis. Upon entry, the signal peptide is cleaved and chaperone-mediated folding of soluble proteins occurs (Vitale and Denecke, 1999; Trombetta and Parodi, 2003); misfolded proteins are identified and translocated back across the ER membrane to the cytosol for degradation (Brandizzi et al., 2003). The ER lumen is a site for *N*-glycosylation of soluble and membrane proteins. ER-resident proteins are retained in the ER or recycled back to the ER from the Golgi by an H/KDEL C-terminal signal on these proteins (Vitale and Denecke, 1999). From the ER, vesicles bud out and fuse with the *cis* face of the Golgi. At the Golgi, sorting and delivery of cargo proteins to multiple destinations takes place (Dupree and Sherrier, 1998; Gu et al., 2001) along with further glycosylation (Hawes and Satiat-Jeunemaitre, 2005). The Golgi is subdivided into stacks making the *cis*-, medial, and *trans*- cisternae, classified based on their enzyme activity. At the

trans side of the Golgi is the *trans*-Golgi network (TGN) where critical sorting and delivery to specific destinations occurs. Sorting signals and motifs on cargo molecules are recognized by vesicle coat components for delivery to their target destinations (Gu et al., 2001). In addition, the TGN can receive extracellular material and recycled components from endosomes (Maxfield and McGraw, 2004), membrane-bound compartments that are derived from the endocytic process. Vesicles bud from the PM and fuse to form endosomes. These endosomes sort membrane cargo and receptors from the PM and the extracellular environment inside the cell for subsequent use by the cell, recycling or degradation. Some of the endocytosed material is eventually passed to the vacuole/lysosome via the late endosomes/PVC (Samaj et al., 2005).

Several events are required for routine maintenance of the cells and organelles. First, new proteins have to be synthesized and shipped to their appropriate destinations. Second, damaged components have to be exchanged and recycled with newer ones to maintain efficiency of tasks. Membranes involved in retrograde or anterograde trafficking have to continuously be shuffled between organelles for continual function. The biosynthetic system traffics vesicles from the ER to the Golgi and then these vesicles are sorted to the PM or vacuole through budding from one organelle and fusing with the next (Sanderfoot and Raikhel, 1999). Three major trafficking pathways can be generalized: [1] ER-Golgi trafficking; [2] Post-Golgi trafficking; [3] Endosomal trafficking and endocytosis (Figure 1).

ER-Golgi Trafficking. All proteins synthesized at the ER are predestined for exocytosis, transport to intracellular compartments or retention in the ER (Figure 1). The biosynthetic pathway trafficks proteins from the ER through the Golgi stacks on their way to

the plasma membrane or vacuole (Jürgens, 2004). Plant cells have several to hundreds of Golgi stacks (Staehelin and Moore, 1995) which are often associated with the ER (Hawes and Satiat-Jeunemaitre, 2005). Together the ER and Golgi make a dynamic system for molecule exchanges (Brandizzi et al., 2002). These exchanges can be disrupted by the fungal metabolite Brefeldin A (BFA), which has been used extensively to determine the mechanism of ER-Golgi transport. BFA disrupts ER to Golgi transport, causing the redistribution of the *cis*-Golgi into the ER without affecting the TGN (Fujiwara et al., 1988; Klausner et al., 1992; Nebenführ et al., 2002). These ER-Golgi hybrid compartments thus formed, termed BFA bodies (Orci et al., 1993), are used to study the ER-Golgi interface, and its disruption is reversible upon removal of BFA. Recent evidence also shows that BFA effects can extend to the PVC and can be used to study Golgi and PVC relations (Tse et al., 2006). BFA treatment along with Golgi photobleaching studies have led to the suggestion that formation of new Golgi requires Golgi-derived COPI membranes and ER-derived COPII membranes (Ward and Brandizzi, 2004). COPI and COPII are coat protein complexes which are necessary for budding of vesicles that transport cargo between the ER and Golgi. COPII vesicles mediate anterograde trafficking from ER-to-Golgi, with SAR1 GTPase, Sec23/24 heterodimer, and Sec13/31 heterotetramer initiating vesicle formation (Barlowe, 2002; Bi et al., 2002; Lee and Miller, 2007). COPI vesicles along with ADP-ribosylation factor 1 (ARF1) GTPase mediate retrograde trafficking from Golgi to ER (Palmer et al., 1993; Spang, 2002). Finally cargo delivery to the Golgi is mediated by Rab1 GTPase and soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) which control vesicle fusion at the Golgi (Allan et al., 2000; Neumann, 2003; Lupashin and Sztul, 2005; Markgraf et al., 2007).

Post-Golgi Trafficking. Protein exiting the Golgi is en-route to the PM and the vacuolar system (Figure 1). The Golgi is a major transportation hub from which trafficking pathways diverge. The default destination of complex carbohydrates and proteins without sorting information is secretion to the PM (Denecke et al., 1990; Hadlington and Denecke, 2000). The presence of sorting signals is essential for transport to the vacuole. The plant vacuolar system consists of multiple types of vacuoles (Jauh et al., 1998; Jiang and Rogers, 1998; Neuhaus and Rogers, 1998; Hinz et al., 1999; Matsuoka, 1999; Paris et al., 2001) distinguished by distinct sets of TIPs (tonoplast intrinsic proteins), luminal content and specific targeting signals (Jauh et al., 1998; Jauh et al., 1999). Lytic vacuoles (LV) are defined by γ-TIP, while protein storage vacuoles (PSV) in vegetative tissues are defined by the presence of δ-TIP, and combinations of δ-TIP plus α- or γ-TIP can be seen in seed PSVs (Jauh et al., 1999; Moriyasu et al., 2003). Evidence now suggests that vacuoles ultimately form a single hybrid vacuole that contains both α- and γ-TIP markers; α-TIP is replaced by an increasing amount of γ-TIP as the vacuole develops (Hunter et al., 2007; Olbrich et al., 2007).

Transport to the vacuole takes place through two pathways after exiting the Golgi: (1) a clathrin-mediated route to the LV via the PVC or multivesicular bodies that employs a sorting receptor (Shimada et al., 2003; Hinz et al., 2007); and (2) a dense vesicle route (Hinz et al., 1999). In an alternative pathway, ER proteins bypass the Golgi and are transported directly to the vacuole via precursor-accumulating vesicles (Hara-Nishimura et al., 1998; Mitsuhashi et al., 2001). In the receptor-mediated pathway via the PVC, receptors are salvaged by recycling back to the TGN from the PVC (daSilva et al., 2005). To enter any of these vacuole-bound pathways, proteins must carry positive sorting information in addition to

an N-terminal sorting signal into the ER (Hadlington and Denecke, 2000). Vacuolar sorting signals (VSS) that these proteins carry are grouped into three groups: (1) sequence-specific VSS (ssVSS) which are N- or C-terminal propeptides or internal sequences that bind vacuolar sorting receptors; (2) C-terminal VSS (ctVSS) which consist of a variable sequence located at the very C-terminal end; and (3) physical-structure dependent VSS (psVSS) which function by aggregate formation (Matsuoka, 1999; Jolliffe et al., 2005).

Endosomal Trafficking and Endocytosis. The secretion machinery continuously adds vesicle-derived membranes to the PM (Figure 1). To balance the increasing membrane surface, membrane is internalized from the PM by clathrin-coated endocytic vesicles (Battey et al., 1999; Holstein, 2002). These internalized vesicles fuse to form what are termed endosomes, organelles that transport recycled protein and membranes towards the Golgi or towards the vacuole for degradation, in addition to regulating the protein content at the PM (Gruenberg and Maxfield, 1995; Neumann, 2003; Samaj et al., 2005). The reorganization of PM protein distribution provides an important way to sense and responded to environmental conditions such as in the case of auxin changes mediated by PIN proteins (Levine, 2002; Paciorek et al., 2005; Dhonukshe et al., 2007).

Internalized vesicles fuse to form endosomes as they enter the cell. Endosomes mature to early/recycling endosomes or sorting endosomes and become late endosomes as their internal pH decreases (Mellman, 1996; Sanderfoot and Raikhel, 2003; Maxfield and McGraw, 2004). There are several events that can occur at early/recycling endosomes. First, early endosomes may recycle PM constituents back to the PM. Other components that may be damaged, misfolded or ubiquitinated are sent to sorting endosomes for further sorting. Second, these endosomes mature to late endosomes, which may be equivalent to prevacuolar

or prelysosomal compartments, and their cargo is either passed towards the vacuole or to the TGN. Third, PM cargo and receptors in endosomes are trafficked towards the TGN and from there, cargo can be sent back to early/recycling endosomes towards the PM or to late endosomes for traffic toward the vacuole (Betz et al., 1996).

Endocytosed proteins pass through several endosomal compartments as they travel towards their final destinations. In studying the pathway of endocytosis, fluorescent markers are used to visualize active movement inside the cell and uptake into the cell. Some of the best characterized fluorescent markers are amphiphilic styryl dyes developed by Betz and coworkers that are able to fluoresce when in the hydrophobic environment of the membrane (Betz et al., 1996). These dyes, FM4-64 and FM1-34, are now commonly used in studying vesicle trafficking and organelle organization in various cells (Betz et al., 1996; Cochilla et al., 1999; Bolte et al., 2004).

Phosphoinositides

Lipids are important components of the cell. Their functions range from being involved in signaling (Simons and Toomre, 2000; Munnik, 2001; Wang, 2004; Eyster, 2007), being a source of metabolic energy (Murphy, 1990, 1993; Voelker and Kinney, 2001), freezing-stress responses (Welti et al., 2002), plant defense (Mehdy, 1994; García-Olmedo et al., 1995; Laxalt and Munnik, 2002) to being structural components of the cell membranes or of organelles inside the cell. The characteristic feature of all membrane lipids is that they contain a hydrophobic and hydrophilic constituent. Membranes establish semi-permeable barriers in the cell and also function in providing a platform for assembly and function of enzymes and macromolecules, and act as molecular signals for metabolic events.

Phospholipids are the most abundant component of most membranes in the cell. The main phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and sphingomyelin. Of the total membrane lipid, phosphoinositols and phosphoinositol phosphates make up at least 5.5% of the total lipids in Arabidopsis (Bonaventure et al., 2003). Phosphatidylinositols or phosphoinositides are key molecules inside the cell. They are versatile as they can serve both as precursors of secondary messengers and as regulators of protein targets by direct binding (Martin, 1998; Hurley and Misra, 2000; Niggli, 2005). Phosphatidylinositol lipids are distinguished easily from other phospholipids in the plasma membrane, intracellular organelles and transport vesicles by the presence of their inositol group. These lipids contribute to the generation of organelle identity through recruitment of specific sets of proteins with appropriate phosphoinositide-binding properties (Meijer and Munnik, 2003). Phosphoinositides can be interconverted between their different derivatives by single or multiple phosphorylations at the D3, D4 and D5 sites on their inositol ring by phosphoinositide kinases or phosphatases (Fruman et al., 1998). Phosphoinositide species can be further converted by modification enzymes like phosphatases, kinases and phospholipases. Intracellular localization and activity of the modifying enzymes reveals the localization and nature of the phosphoinositide. The polarized localization and change in phosphoinositide profile significantly influences the localization and activity of the proteins that bind specific phosphoinositides to produce downstream signaling events (Itoh and Takenawa, 2002).

Phosphoinositides reside on the cytoplasmic side of membranes and control the membrane-cytosol interface (Corvera, 2001; Itoh and De Camilli, 2006). A phosphatidylinositol derivative defines the membrane/organelle characteristics, identity

and/or location. A change in phosphorylation of these derivatives can result in an alteration of the function at that membrane (Balla and Varnai, 2002; Meijer and Munnik, 2003; Halet, 2005; Vermeer et al., 2006).

Phosphatidylinositols (PIs or PtdIns) are synthesized at the endoplasmic reticulum (Imai and Gershengorn, 1987; Antonsson, 1997; Collin et al., 1999) and eventually become phosphorylated or dephosphorylated to any of the following derivatives: [1] phosphatidylinositol 3-phosphate (PtdIns3P); [2] phosphatidylinositol 4-phosphate (PtdIns4P); [3] phosphatidylinositol 5-phosphate (PtdIns5P); [4] phosphatidylinositol 3,4-bisphospate ($PtdIns(3,4)P_2$); [5] phosphatidylinositols 3,5-bisphosphate ($PtdIns(3,5)P_2$); [6] phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$); and lastly [7] phosphatidylinositol 3,4,5-trisphosphate ($PtdIns(3,4,5)P_3$). All seven variations have been found in animals but only six in plant cells (not phosphatidylinositol (3,4,5)-trisphosphate) (Mueller-Roeber and Pical, 2002).

Intracellular Distribution of Phosphoinositides. Phosphoinositides are important factors in membrane trafficking. The sites of phosphoinositides in the cell have been inferred from the locations of proteins involved in making, altering or binding these lipids, and additional information is acquired by visualization using lipid binding domains fused with fluorescent proteins (Balla and Varnai, 2002; Meijer and Munnik, 2003; Halet, 2005; Vermeer et al., 2006). However, these methods have several limitations. These visualization methods target free pools of phosphoinositides and not those which are already bound to proteins (Balla et al., 2000). Expression of lipid-binding fusion protein at physiological levels is insufficient for visualization of phosphoinositides while high levels interfere with phosphoinositide production, regulation, and function. These methods can only yield

information on uncomplexed phosphoinositides; active-phosphoinositide complexes cannot be examined with the current methods. Much locational information of phosphoinositides is derived from mammalian models; however, even though less information can be found for plants, it can be assumed that plants have similar locations and functions of these phosphoinositides. Locations of phosphoinositides are as follows: PtdIns(3)P is the most abundant and is present primarily on endosomal membranes (Burd and Emr, 1998; Gagescu, 2000; Gillooly et al., 2000); PtdIns(4,5)P_2 is at the plasma membrane (Holz et al., 2000; Martin, 2001; Holz and Axelrod, 2002; Lemmon, 2003; Roth, 2004; Takenawa and Itoh, 2006), Golgi (Roth, 2004) or nucleus (Osborne et al., 2001) and on internal membranes enriched in lipid rafts (Rozelle et al., 2000; Brown et al., 2001); PtdIns(4)P is on the Golgi (Balla and Varnai, 2002; Burda et al., 2002; Wang et al., 2003; Roth, 2004; Takenawa and Itoh, 2006). PtdIns(3,5)P_2 is present in multivesicular bodies/endosomes (Gary et al., 1998; Wurmser and Emr, 1998; Friant et al., 2003; Hicke, 2003; Shaw et al., 2003; Roth, 2004) and on the vacuole/lysosomes (Gary et al., 1998; Wurmser and Emr, 1998; Hicke, 2003). PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 are mostly involved as secondary messengers in signaling at the plasma membrane and nucleus (Takenawa and Itoh, 2006; Sasaki et al., 2007). Lastly, PtdIns(5)P is present at the plasma membrane and the nucleus (Jones et al., 2006).

Regulation by Phosphoinositides. Phosphoinositides are universal lipid regulators of protein signaling complexes. Phosphoinositides present a reversible signaling system. Being bound to a membrane and possessing sites for multiple phosphorylation combinations, these lipids can easily switch among phosphorylation states, affecting function and signaling (Stevenson et al., 2000; Meijer and Munnik, 2003). Phosphorylated lipids interact with proteins that can bind these lipids and allow the recruitment of proteins to the membrane

(Meijer and Munnik, 2003). Phosphoinositide-binding proteins remain in an inactive state in the cytosol and are attracted to membranes upon production of the appropriate phosphoinositide species. These proteins remain active on the membrane and recruit additional proteins; control can be fine tuned by lipid kinases and phosphatases present at the membrane (van der Geer and Pawson, 1995; Balla, 2005; Konig et al., 2007).

Phosphoinositide-Binding Domains

Several structural domains bind phosphoinositides. These domains are involved in targeting proteins to the membrane. In some cases, additional protein-protein interaction domains are present to support phosphoinositide-binding domains. There are several phosphoinositide-binding domains that work alone, as dimers, or in conjunction with other protein interaction domains. These include: PH domain; PTB domain; FERM domain; FYVE domain; PDZ domain; ENTH domain; ANTH domain; and PX domain. Although these domains were discovered in mammalian cells, they can be found in bacteria, yeast and plants as well.

PH, PTB and FERM domains. The Pleckstrin Homology (PH) domain is the best characterized phosphoinositide-binding domain, with over 100 different PH domain-containing proteins known (Bottomley et al., 1998). The domain, first identified in a protein kinase C substrate called pleckstrin, is a 100-120 amino acid domain contained in proteins that are involved in intracellular signaling and cytoskeleton organization (Harlan et al., 1994; Itoh and Takenawa, 2002; Takenawa and Itoh, 2006). PH domains are divided into four groups based on the phosphoinositide with which the domain associates. Group 1 solely binds PtdIns(3,4,5)P_3; Group 2 binds mostly PtdIns(4,5)P_2 in addition to binding to other

ligands; Group 3 specifically binds PtdIns(3,4)P_2 ; and Group 4 has no specific binding specificity (Itoh and Takenawa, 2002). PH domains recruit proteins to the membrane in a phosphoinositide-dependent manner to form larger complexes which are involved in Ras, PI3K and G-protein signaling pathways (Bottomley et al., 1998; Itoh and Takenawa, 2002). Phosphotyrosine-binding (PTB) domains resemble the PH domain and were initially identified as domains for recognition for phosphorylated Asn-Pro-Xxx-pTyr-containing proteins (Forman-Kay and Pawson, 1999; Lemmon et al., 2002). The PTB domain functions in signal transduction and is present in adaptor proteins interacting adjacent to tyrosine kinase receptors (van der Geer and Pawson, 1995; Balla, 2005). FERM domains contain a subdomain C showing highly conserved PTB structural folds which resembles the PH/PTB domain (Balla, 2005). FERM (protein 4.1, Ezrin, Radixin, Moiesin) discovered in proteins linking the actin cytoskeleton to the plasma membrane (Chishti et al., 1998). Although these domains were discovered in mammalian systems, evidence suggests plant proteins also contain similar conserved domains (Chen et al., 1998). The functions of these domains in plants are not well characterized but are likely related in function to animals.

PDZ domain. Named after three proteins which contain them, brain-specific protein PSD-95, the Drosophila septate junction protein Disks-large, and the epithelial tight-junction protein ZO1, PDZ domains consist of ~85 amino acid residues which generally bind to the peptide motif X-Ser/Thr-X-Val at the C-terminal end of transmembrane proteins, in addition, to binding to phosphoinositides (Harrison, 1996; Doyle et al., 2004). These domains usually organize with additional PDZ domains or other modular domains to form protein signaling complexes at specific cellular regions (Nourry et al., 2003).

FYVE domain. First identified as a zinc finger motif in early endosomal antigen 1 (EEA1), FYVE got its name from four proteins, namely Fab1p, YOTB, Vac1p, and EEA1 (Stenmark et al., 1996). The FYVE finger requires two Zn^{2+} ions and a conserved PtdIns3P - binding site (R/S R/K H/Y H/R C R/K) that functions in the membrane recruitment of cytosolic proteins (Itoh and Takenawa, 2002; Balla, 2005). FYVE-containing proteins exclusively bind PtdIns3P and have been shown to be involved in endosome and vacuolar sorting, endocytosis, growth factor signaling and regulation of the actin cytoskeleton (Kutateladze et al., 1999; Wurmser et al., 1999; Corvera, 2000; Taunton et al., 2000; Kutateladze and Overduin, 2001; Itoh and Takenawa, 2002; Balla, 2005).

ENTH and ANTH domains. Epsin N-terminal homology (ENTH) domain is a 140 amino acid conserved region found in proteins associated with clathrin-mediated endocytosis or vesicle budding (Kay et al., 1999; Legendre-Guillemin et al., 2004). It was first identified in epsin, a factor involved in the budding process of clathrin-coated vesicles during endocytosis (Chen et al., 1998). The ENTH domain binds PtdIns(4,5)P_2 (Ford et al., 2001; Itoh et al., 2001). Proteins related to ENTH domain-binding proteins are found to have a structurally distinct amino terminal domain (ANTH domain) that binds the phosphoinositide-head group instead of the lipid in the case of the ENTH domain (Ford et al., 2001; Ford et al., 2002). ENTH domain binding of PtdIns(4,5)P_2 causes membrane dissociation of the protein and induction of membrane curvature, while ANTH domain proteins bind to clathrin and AP2 adaptors to cause the polymerization of the clathrin coat (Ford et al., 2002; Stahelin et al., 2003). ENTH/ANTH protein domains contain multiple motifs that allow the formation of a scaffold for protein complex assembly on the membrane (Kalthoff et al., 2002). In plants, ENTH/ANTH domains have been shown to be involved in mediating clathrin vesicle

formation and implicated in plant protein trafficking (Holstein and Oliviusson, 2005; Song et al., 2006; Lee et al., 2007).

PX domain. The PX or phox (phagocyte oxidase) homology domain is a 100-120 amino acid domain first identified in two cytosolic components of neutrophil NADPH oxidase complex, $p47^{phox}$ and $p40^{phox}$ (Wientjes et al., 1993; Ponting, 1996), and later identified as binding to 3-phosphoinositides (Ellson et al., 2001; Kanai et al., 2001). This domain, which is conserved among numerous proteins including NADPH oxidase, phospholipase and the sorting nexin protein family, increases the affinity of proteins for the membranes allowing them to function in protein sorting, vesicle trafficking, and phospholipid metabolism and signaling (Horazdovsky et al., 1997; Sato et al., 2001). The sorting nexin protein family is an essential key to understanding PX domains being an evolutionarily conserved domain and with increasing evidence for its role in endosomal trafficking pathways (Roth, 2004).

Sorting Nexins

Sorting nexins (SNXs) are membrane-associated proteins that are involved in various aspects of endocytosis and protein trafficking through phosphoinositol lipid-containing organelles (Worby and Dixon, 2002). SNXs are a large family of proteins containing a PX homology domain which binds phosphatidylinositol phosphates (Teasdale et al., 2001). This family of proteins shows limited sequence conservation and a wide range of phosphatidylinositol phosphate-binding specificities. The first sorting nexin, SNX1, was identified through a yeast two-hybrid screen for binding to EGF receptor (Kurten et al., 1996) and subsequently other sorting nexins have been discovered to associate with membrane

receptors to regulate receptor recycling and degradation (Horazdovsky et al., 1997; Haft et al., 1998; Stockinger, 2002). All sorting nexins are united by the presence of a PX domain and though not all have been studied in detail, their functions are very diverse. To date, there have been 29 mammalian SNXs and 10 yeast SNXs described (Carlton et al., 2005).

In addition to the PX domain, SNXs also possess other domains that aid in protein-protein interaction and protein-lipid association (Teasdale et al., 2001). A strong tendency to form coiled-coil-mediated protein complexes makes them likely candidates for control of multiple trafficking pathways (Verges, 2007). SNXs self-assemble to form homodimers and heterodimers (Haft et al., 2000; Kurten et al., 2001). The dimerization utilizes known and predicted domains which work jointly with the PX domain for protein-protein interaction; these domains include the Src homology 3 domain (SH3), regulator of G-protein signaling domain (RGS), PX associated domain (PXA), microtubule interacting domain (MIT), PDZ domain (PDZ), RhoGAP domain, Ras associated domain (RA), kinesin motor and the more recently discovered BAR (Bin/Amphiphysin/Rvs) domain (Carlton et al., 2005).

Among the different interacting domains in SNXs, the BAR domain is predicted to be present in a subset of sorting nexins (Carlton et al., 2004; Carlton et al., 2005). The BAR domain acts as a sensor for membrane curvature and allows the protein to recognize and induce curvature on membranes (Habermann, 2004; Miele et al., 2004; Peter et al., 2004; Zimmerberg and McLaughlin, 2004). Studies by Carlton et al. (2004) have shown that the C-terminal BAR domain on mammalian SNX1 determines membrane recognition and binding. The BAR domain along with the PX domain has the potential to allow the sorting nexin itself to recognize specific targeted membranes effectively.

Yeast Sorting Nexins. The functions of two yeast SNXs, Vps5 and Vps17, are well understood. Vps5, a yeast homolog of mammalian SNX1 and SNX2, was identified as a protein involved in retrieval of cargo away from vacuole-mediated degradation (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997; Nothwehr et al., 2000; Pelham, 2002). This yeast sorting nexin is specifically involved in sorting of Vps10p, the receptor for the soluble vacuolar hydrolase carboxypeptidase Y (CPY), the Golgi resident membrane protein A-ALP, and endopeptidases Kex2p and dipeptidyl amino peptidase (DPAP) (Nothwehr and Hindes, 1997; Carlton et al., 2005). Vps5 binds PtdIns(3)P through its PX domain and this allows Vps5 to localize to endosomal membranes (Burda et al., 2002). Vps5p and Vps17p dimerize to bind the Vps26/Vps29/Vps35 cargo selection complex, making what is called the 'retromer complex' that mediates endosome-to-Golgi retrieval of Vps10p (Seaman et al., 1998; Nothwehr et al., 2000; Seaman and Williams, 2002). The yeast retromer also functions with yeast SNX Grd19/Snx3p as a cargo-specific adaptor to transit the iron transporters, Fet3 and Ftr1, from endosomes to the Golgi for re-secretion (Strochlic et al., 2007). Grd19/Snx3p interaction with the retromer complex established a mechanism by which sorting nexins can expand the retromer cargo repertoire (Strochlic et al., 2007).

Mammalian Sorting Nexins. SNX1, a partially membrane-associated protein, was first identified as a yeast two-hybrid partner with epidermal growth factor receptor (EGFR) (Kurten et al., 1996). It was later found to associate with endosomes (Kurten et al., 2001; Cozier et al., 2002; Zhong et al., 2002) and enhance EGFR degradation upon overexpression. However, recent studies using RNA interference (RNAi) technology to suppress SNX1 expression suggest that endogenous SNX1 plays no direct role in epidermal growth factor or transferrin recycling (Carlton et al., 2004; Gullapalli et al., 2004). SNX1 suppression,

however, causes a defect in the retrieval of cation-independent mannose-6-phosphate receptor (CI-M6PR) (equivalent to Vps10) from sorting endosomes by the mammalian retromer complex (Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004). SNX1 has been suggested to form complexes with mammalian SNX2 (Haft et al., 2000). Both mammalian SNX1 and SNX2 are localized together to the early endosomes and play separate roles in lysosomal sorting of internalized EGFR (Zhong et al., 2002; Gullapalli et al., 2004). SNX1 and SNX2 have been implicated as components of the mammalian retromer complex.

Additional mammalian sorting nexins SNX3, SNX4, SNX5, SNX10, SNX15 and SNX16 have been found to have functions related to endosomal morphology or transport (Barr et al., 2000; Xu et al., 2001; Hanson and Hong, 2003; Merino-Trigo et al., 2004; Qin et al., 2006; Traer et al., 2007). Little is known about the specific functions of other mammalian sorting nexins.

Plant Sorting Nexins. Sorting nexins in plants have not been studied in great detail. The first indication of sorting nexins functioning in plants was presented by Vanoosthuyse and coworkers in *Brassica oleracea* (Vanoosthuyse et al., 2003). *Brassica oleracea* sorting nexin (BoSNX1) was identified in a yeast two-hybrid screen as an interactor with *S* locus receptor kinase, a protein involved in the self-incompatibility response in pollen self recognition (Vanoosthuyse et al., 2003).

Arabidopsis has three identified sorting nexins, AtSNX1, AtSNX2a and AtSNX2b (Vanoosthuyse et al., 2003). The analysis of a BoSNX1 homolog in Arabidopsis identified AtSNX1. AtSNX1 was found to localize to sorting endosomes and is involved in a novel auxin-related trafficking pathway defined by auxin efflux carrier PIN2 (Jaillais et al., 2006;

Jaillais et al., 2008). An *Atsnx1* null mutant exhibited auxin-related defects and enhanced the phenotype of a weak mutant of GNOM, an ARF GTPase involved in vesicle coat formation and trafficking (Jaillais et al., 2006). The sorting of PIN2 by an AtSNX1-positive endosome represents a transport pathway that probably involves the Arabidopsis retromer complex (Jaillais et al., 2006; Jaillais et al., 2007).

Importance of the Retromer Complex. Trafficking and signaling involve similar core components. The components of vesicle coats such as clathrin, COPI, and COPII are important in directing a vesicle to its correct intracellular destination in addition to being involved in cargo selection and budding (Cai et al., 2007; Mills, 2007). The retromer complex recycles components from the prevacuolar compartment to the TGN (Seaman et al., 1997; Seaman et al., 1998; Pfeffer, 2001; Oliviusson et al., 2006) and is believed to be a novel vesicle coat distinct from clathrin, COPI and COPII. In general, depending on the organism, the multimeric complex comprises several eukaryotic homologs of the yeast heterotrimer VPS26–VPS29–VPS35 and SNXs VPS5 and VPS17 (Heinzerling et al., 2004; Seaman, 2004; Griffin et al., 2005; Oliviusson et al., 2006; Shimada et al., 2006; Gokool et al., 2007; Hierro et al., 2007; Jaillais et al., 2007; Yamazaki et al., 2008). The control of receptor trafficking at endosomes has been attributed to the retromer complex (Seaman, 2004; Seaman, 2005; Jaillais et al., 2006; Oliviusson et al., 2006; Shimada et al., 2006; Seaman, 2007; Verges, 2007; Jaillais et al., 2008). The control of receptor sorting by retromer and SNX is necessary for maintaining cellular proliferation and equilibrium (Verges, 2007). Work in yeast, plants and mammals has identified analogous conserved functions of the retromer complex in endosomal retrieval of receptors, however other sorting receptors as a cargo of the retromer and the ligands of the receptors remain to be identified in

higher eukaryotes. The identification and characterization of the cargos of the retromer and the ligands of the receptors which are trafficked by the retromer complex are critical for future studies investigating the role of the complex in growth and development.

Conclusion and Dissertation Outline

The overall focus of this dissertation research is to study the function of *Arabidopsis thaliana* sorting nexin 2b (AtSNX2b). Presented here are the cellular and *in planta* roles of Arabidopsis sorting nexin 2b. Three chapters summarizing results from these studies in *A. thaliana* will be presented. In Chapter 2, intracellular functions of Arabidopsis AtSNX2b will be addressed. Chapter 3 summarizes phenotypic effects in *Arabidopsis Atsnx2b* mutants. In Chapter 4, proteins interacting with AtSNX2b will be discussed. The final chapter in this dissertation summarizes the conclusions and addresses future experiments that are needed to better understand the topics presented.

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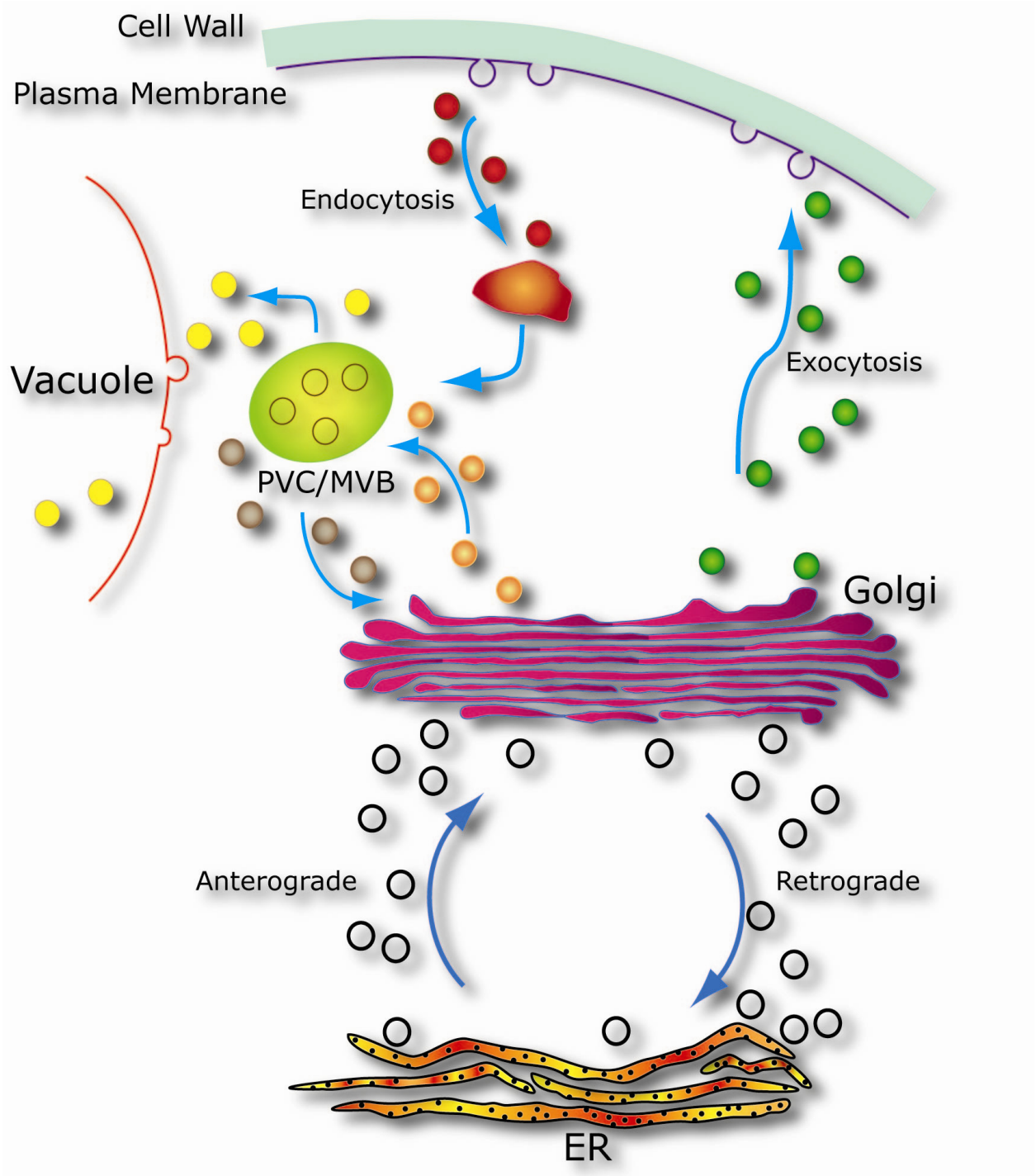

Figure 1. Overview of protein trafficking in plants. Shown is a diagram of protein transport pathways in the plant cell. Abbreviations: ER – endoplasmic reticulum; PVC/MVB - prevacuolar compartment/multivesicular bodies.

CHAPTER 2. OVEREXPRESSION OF ARABIDOPSIS SORTING NEXIN AtSNX2b INHIBITS ENDOCYTIC TRAFFICKING TO THE VACUOLE

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Abstract

Sorting nexins are conserved proteins that function in vesicular trafficking and contain a characteristic phox homology (PX) domain. Here we characterize the ubiquitously-expressed *Arabidopsis thaliana* sorting nexin AtSNX2b. Subcellular fractionation studies indicate that AtSNX2b is peripherally associated with membranes. The AtSNX2b PX domain binds to phosphatidylinositol 3-phosphate *in vitro* and this association is required for the localization of AtSNX2b to punctate structures *in vivo,* identified as the *trans*-Golgi network, prevacuolar compartment and endosomes. Overexpression of GFP-tagged AtSNX2b produces enlarged GFP-labeled compartments that can also be labeled by the endocytic tracer FM4-64. Endocytic trafficking of FM4-64 to the vacuole is arrested in these GFP-AtSNX2b compartments, and similar FM4-64-accumulating compartments are seen upon overexpression of untagged AtSNX2b. This suggests that exit of membrane components from these enlarged or aggregated endosomes is inhibited. AtSNX2b overexpression interferes with the transport to the vacuole of proteins containing an N-terminal propeptide,

but has no effect on transport of proteins containing a C-terminal propeptide. We hypothesize that AtSNX2b is involved in vesicular trafficking from endosomes to the vacuole.

Introduction

The structure and function of cellular organelles is maintained by a network of pathways for protein synthesis, sorting, recycling, regulation and degradation. In the endomembrane system, new proteins are synthesized on membrane-bound ribosomes at the endoplasmic reticulum (ER). These proteins are folded and glycosylated before being transported to the Golgi where they can be further modified as they move from the *cis* to the *trans* cisternae. At the *trans* face of the Golgi resides the *trans*-Golgi network (TGN), where critical sorting events take place to target proteins either to the cell surface or to the vacuole. Transport to the plasma membrane may occur via a default pathway (Batoko et al., 2000; Mitsuhashi et al., 2000), while vacuolar proteins bind to specific receptors for sorting to their final destinations (Kirsch et al., 1994; Ahmed et al., 2000; Shimada et al., 2003; Park et al., 2005). However, some secreted material, for example cell wall components, is deposited in a polarized manner (Freshour et al., 2003; Lee et al., 2005), and some proteins show a polarized distribution in the plasma membrane (Dhonukshe et al., 2005), suggesting that default secretion is an oversimplification.

In plants, the vacuole is a multifunctional organelle that is essential for plant survival. Vacuolar functions include storage, degradation, sequestration of xenobiotics, maintenance of turgor and protoplasmic homeostasis (Marty, 1999). Whereas in some cells these functions may reside within a single central vacuole, in at least some cell types multiple functionally distinct vacuoles are present that can be distinguished by their unique protein composition

(Paris et al., 1996; Di Sansebastiano et al., 2001; Olbrich et al., 2007). Delivery of proteins to the vacuole in plants involves two general pathways: [1] the biosynthetic route via ER and Golgi compartments and [2] the endocytic route via endocytosis from the cell surface (Chrispeels and Raikhel, 1992; Marty, 1999; Matsuoka and Neuhaus, 1999; Hanton and Brandizzi, 2006). Three types of signal have been identified for targeting proteins to the plant vacuole via the biosynthetic route, a C-terminal propeptide (CTPP), a sequence-specific vacuolar sorting signal, usually in the form of an N-terminal propeptide (NTPP), and an internal signal (Marty, 1999). These signals are recognized at the TGN by vacuolar sorting receptors for correct targeting to the lytic or storage vacuole (Ahmed et al., 2000; Paris and Neuhaus, 2002; Shimada et al., 2003; Jolliffe et al., 2005). Distinct pathways, and possibly different receptors, exist for transport to different vacuole types, and some storage proteins are even transported directly from the ER to the vacuole, bypassing the Golgi apparatus entirely (Hillmer et al., 2001; Shimada et al., 2003; Park et al., 2005).

Endocytic trafficking to the plant vacuole begins at the plasma membrane, where membrane and extracellular cargo are internalized either by clathrin-coated vesicles or via a clathrin-independent pathway (Grebe et al., 2003; Baluska et al., 2004; Holstein and Oliviusson, 2005). These vesicles probably fuse with endosomes, organelles that function in sorting, recycling and further transport of cargo and membrane (Samaj et al., 2005; Muller et al., 2007).They can be classified into several distinct types based on their protein content, and by functional criteria (Ueda et al., 2004; Yamada et al., 2005; Dettmer et al., 2006; Haas et al., 2007; Lam et al., 2007; Jaillais et al., 2008). The biosynthetic and endocytic transport pathways probably merge at late endosomes, which may be equivalent to the prevacuolar compartment (PVC), from which point both pathways utilize similar trafficking components

to aid routing of cargos to the vacuole (Samaj et al., 2005; Yamada et al., 2005; Muller et al., 2007; Jaillais et al., 2008).

Increasing evidence indicates a role for the sorting nexin family of lipid-binding proteins in protein trafficking and sorting. Sorting nexins (SNXs) are membrane-associated proteins that are involved in endocytosis and protein trafficking through phosphatidylinositol lipid-containing organelles and are conserved in eukaryotes (Worby and Dixon, 2002; Carlton et al., 2005). SNXs are part of a large family of proteins that are defined by the presence of a phox homology domain (Teasdale et al., 2001), a 100-130 amino acid domain that binds phosphatidylinositol (PI) phosphates and targets the proteins to specific cellular membranes enriched in that phospholipid. In mammals, SNX functions include recycling or degradation of receptors (Kurten et al., 1996), control of endosome morphology (Barr et al., 2000), and regulation of endosomal function (Xu et al., 2001; Rojas et al., 2007). Several lines of evidence have implicated SNXs in an endosome-to-Golgi retrograde trafficking pathway. Genetics screens in yeast have identified a pentameric retromer complex required for the transport of proteins, including the sorting receptor Vps10p, from the PVC to the Golgi complex (Seaman et al., 1997; Seaman et al., 1998). Vps5p and Vps17p are SNXs that dimerize (Horazdovsky et al., 1997) to mechanically aid in vesicle budding while Vps35p and Vps29p act as cargo selectors (Seaman et al., 1998; Reddy and Seaman, 2001). Lastly Vps26p binds the complex together (Reddy and Seaman, 2001). Recent work from several laboratories has provided evidence of retromers in other model organisms and identified homologs of the yeast genes *VPS29*, *VPS30* and *VPS35* in Arabidopsis and mammals (Haft et al., 2000; Oliviusson et al., 2006; Shimada et al., 2006; Jaillais et al., 2007; Yamazaki et al., 2008). These homologs show similarity to the yeast counterparts and assemble into a

macromolecular complex that is presumed to similarly function in endosome-to-Golgi transport/recycling. Among the better characterized SNXs, mammalian SNX1 and 2, orthologs of Vps5p, have been shown to play a role in the mammalian retromer complex (Haft et al., 2000; Rojas et al., 2007). Other mammalian SNXs are involved in endocytic trafficking and endocytosis of plasma membrane receptors (Parks et al., 2001; Xu et al., 2001; Leprince et al., 2003; Lundmark and Carlsson, 2003; Merino-Trigo et al., 2004).

Initial evidence for a function of SNXs in plants came from a yeast-two hybrid assay demonstrating that the *Brassica oleracea* S locus receptor kinase intracellular domain interacts with a SNX (*Brassica oleracea* SNX1, BoSNX1) during the self-incompatibility response in pollen recognition (Vanoosthuyse et al., 2003). The closest *BoSNX1* homolog in Arabidopsis is the gene At5g06140 (named *AtSNX1*). AtSNX1 is involved in trafficking of the auxin transport component PIN2 through AtSNX1-containing endosomes via a novel transport pathway (Jaillais et al., 2006) and is probably a component of the Arabidopsis retromer complex (Jaillais et al., 2007). Two additional SNX genes are present in the Arabidopsis genome, designated *AtSNX2a* (At5g58440) and *AtSNX2b* (At5g07120) (Vanoosthuyse et al., 2003). We demonstrate here that the AtSNX2b protein can bind to phosphatidylinositol 3-phosphate (PI3P) and that this interaction is required for its localization to endosomes. Overexpression of AtSNX2b leads to enlargement or aggregation of the AtSNX2b-containing endosomes and interferes with efficient transport to the vacuole. These data implicate AtSNX2b in trafficking of cargo and membrane through an endosomal compartment.

Materials and Methods

Plant materials and growth conditions. *Arabidopsis thaliana* seeds were surface-sterilized in 33% (v/v) bleach and 0.1% (v/v) Triton X-100 solution for 20 min followed by cold treatment of at least 2 d at 4^{o}C. Plants were grown on soil or MS solid medium [Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT), 1% (w/v) Suc (Sigma-Aldrich, St. Louis, MO), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO) and 0.8% (w/v) phytoblend agar (Caisson Lab, Inc., North Logan, UT)] under long-day conditions with ambient light (16 h light; 100µmol $m^{-2}s^{-1}$) at 22°C. *Arabidopsis thaliana* suspension cell cultures were maintained as described by Contento et al. (2005).

A homozygous *Atsnx2b* knockout (GABI_105E07) mutant line was received from the GABI-Kat mutant collection at the Max-Planck-Institute for Plant Breeding Research (Rosso et al., 2003). The T-DNA insertion site was verified by GABI_Kat by sequencing the junction between the T-DNA left border and the AtSNX2b first exon. Additional verification of the mutant allele was done by analysis of segregation of the sulfadiazine resistance marker encoded by the T-DNA insertion by growing seedlings in MS medium containing 12 mg/L (4-amino-*N*-[2-pyrimidinyl]benzene-sulfonamide-Na; GABI-Kat lines).

RT-PCR analysis of AtSNX2b. Total RNA was extracted from Arabidopsis organs using the TRIzol RNA isolation method (http://www.arabidopsis.org/info/2010_projects/comp_proj/AFGC/RevisedAFGC/site2RnaL.htm#isolation) with DNase I treatment. cDNAs were generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using an oligo dT primer. Gene-specific primers were used to amplify AtSNX2b (At5g07120); (forward) 5’-

GGATCCAAAGAAGAGATGGAGAAAC-3’ and (reverse) 5’-GGATCCAATTACACTGTGCTCTCATG-3’. Introduced restriction sites are underlined.

Plasmid Construction. A GFP-AtSNX2b fusion was constructed using a pJ4GFP-XB vector (Igarashi et al., 2001) with modifications as described in Contento et al. (2005). A *BamH*I-*BamH*I fragment consisting of the coding region of the AtSNX2b cDNA (At5g07120) was amplified as above. The *BamH*I-digested fragment was then subcloned into the modified pJ4GFP-XB digested with *Bgl*II. Similarly, a GFP-AtSNX2b-1 mutant fusion construct was made using a three step PCR mutagenesis (Li and Shapiro, 1993) amplifying a 233RR→LG amino acid mutation using mutation primers 5'-GTGGAGCAGCTAGGAGTTGCATTGG-3' (forward) and 5'-CCAATGCAACTCCTAGCTGCTCCAC-3' (reverse) in the first 2 steps followed by amplification of the full AtSNX2b-1 mutant using AtSNX2b specific primers (above) and finally subcloning into the modified pJ4GFP-XB at the same *Bgl*II digested site.

GFP-PX and GFP-PX mutant fusion constructs were made using a similar procedure. A digested *BamH*I-*BamH*I fragment of the PX domain of AtSNX2b (amino acids 142-257) was subcloned into a *Bgl*II-digested pJ4GFP-XB to generate GFP-PX construct. The mutant PX domain (PX-1, amino acid 233RR→LG) was generated using the mutation primers (above) using the three step PCR mutagenesis. A second PX domain mutant (PX-2, amino acid 211PP→AA) was generated through the same three step PCR mutagenesis using primers 5’-CTGCATTGCAGCGAGGCCAGATAA-3’ (forward) and 5’-CTTATCTGGCCTCGCTGCCAATGCAG-3’ (reverse). PX primers used were 5’-GGATCCCCGAATTCCCGGGTCGA-3’ (forward) and 5’-GGATCCTCAGTCACTCAAAGCGGTAACTTCCC-3’ (reverse) for PX, PX-1 and PX-2

constructs. Restriction sites are underlined. The digested *BamH*I-*BamH*I PX fragment was then cloned into *Bgl*II-digested pJ4GFP-XB.

Antibody Production and Purification. The AtSNX2b coding region flanked by *Sal*I-*Not*I restriction sites was generated by RT-PCR from total *Arabidopsis* RNA using the following primers: (forward) 5'-GTCGACTCCCATCTCCACTCATCC-3' and (reverse) 5'-GCGGCCGCATTACACTGTGCTCTC-3' and subcloned into pET28b (Novagen, Madison WI) to produce HIS-AtSNX2b fusion plasmid. The fusion protein was synthesized in *Escherichia coli* according to the Novagen protocol, where it accumulated in inclusion bodies.

HIS-fusions were purified following the manufacturers protocol using HIS·bind resin (Novagen, CAT# 70666). Cells were broken by sonication, and insoluble material was pelleted at 24,000*g*. The pellet was resuspended in 6M urea in HIS binding buffer for 1 h at 4°C and pelleted again by centrifugation at 24,000*g*. After centrifugation, the supernatant was incubated with HIS·bind resin for 30 min and eluted with 100mM imidazole elution buffer. The eluted protein was separated by SDS-PAGE (200 µg), cut from the gel and used to immunize rabbits.

For affinity purification of antibodies, purified HIS-AtSNX2b protein was separated by SDS-PAGE, transferred to nitrocellulose, and the strip containing the fusion protein was cut out after staining with Ponceau S. After blocking in 3% (w/v) dried nonfat milk in PBS, serum was incubated with the strip for 2 h at 4°C to allow binding of the antibodies. The strip was washed with PBS and specific antibodies were eluted using 100 mM Glycine, pH 2.5. The eluate was adjusted to pH 7.0 using 2M Tris-HCl, pH 8. These affinity-purified antibodies were used in all further experiments.

Lipid Overlay Assay. Wild-type PX domain and the mutant PX domain PX-1 were generated by PCR using the primers 5'-GTCGACCGCTCTGATTACATCAAGATC-3' (forward) and 5'-GCGGCCGCTCAAAGCGGTAACTTCCCTTGCG-3' (reverse) from either GFP-PX or GFP-PX-1. *Sal*I-*Not*I fragments of PX and PX-1 were subcloned into pGEX-5X-1 (Amersham Bioscience, Piscataway, NJ) to yield GST-PX and GST-PX-1. GST-PX and GST-PX-1 fusion proteins were synthesized in *E. coli* according to the Novagen protocol. GST-fusions were purified using GST·bind resin (Novagen, CAT# 70541) following the manufacturer's protocol.

Lipid overlay assays were performed according to Dowler et al. (2002). In brief, purified GST-fusions were allows to bind to PIP^{TM} lipid strips (Echelon Biosciences Inc., Salt Lake, UT) followed by immunoblotting using GST antibodies (Invitrogen, Carlsbad, CA) to detect bound GST-fusions.

Transient transformation of Arabidopsis protoplasts. For suspension cells, protoplasts were prepared and transformed according to Contento et al. (2005). For leaf tissue, protoplasts were prepared and transformed according to Sheen (2002). Protoplasts were transformed with 30µg of DNA per transformation. Images were obtained using fluorescence and confocal laser microscopy.

Immunofluorescence. Three-d-old Arabidopsis seedlings grown on MS solid medium were fixed following Sivaguru et al. (1999). Plants were transferred into 5 mL of MTSB buffer (50 mM PIPES-KOH [pH 6.9], 5 mM EGTA, and 5 mM $MgSO_4$) containing 5% (v/v) dimethyl sulfoxide for 15 min at room temperature. Afterward, they were fixed with 4% (w/v) paraformaldehyde in the above buffer containing 10% (v/v) dimethyl sulfoxide for 60 min at 20°C with the initial 10 min under vacuum. Plants were then washed with MTSB prior to

immunostaining. Immunofluorescence staining of treated Arabidopsis seedlings was performed according to Müller et al. (Muller et al., 1998). Treated seedlings were incubated with primary antibodies in a humid chamber for 15-18 h at 4ºC, washed three times for 5 min in MTSB and further incubated for 1–2 h at room temperature with conjugated secondary anti-rabbit or anti-mouse IgG antibodies in 3% (w/v) bovine serum albumin in MTSB. Seedlings were washed five times with MTSB and mounted with a coverslip in 50% (v/v) glycerol in phosphate-buffered saline (PBS, pH 7.4). Primary antibodies used were T7·tag monoclonal antibodies (Novagen/EMD Biosciences, Inc, La Jolla, CA; 1:100), anti-AtSNX2b antibodies (1:200) and preimmune antibodies (for AtSNX2b; 1:200). Secondary antibodies used were Alexa Fluor® 594-conjugated goat anti-rabbit IgG, Alexa Fluor® 488-conjugated goat anti-mouse IgG and Alexa Fluor® 488-conjugated goat anti-rabbit IgG or Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:250).

For protoplasts, transformed protoplasts were fixed in 4% (w/v) paraformaldehyde in MTSB buffer for 20 min followed by 3 washes with MTSBS (MTSB containing 0.4M sorbitol) prior to immunostaining. Protoplast were permeabilized using permeabilization solution (3% (v/v) Triton X-100, 10% (v/v) dimethyl sulfoxide in MTSB) for 20 min. Immunofluorescence staining of fixed protoplasts was performed according to Kang et al. (2001). Cells were washed five times with MTSB and mounted with a coverslip in 50% (v/v) glycerol in PBS. Antibodies used were anti-AtSNX2b antibodies (1:200) , anti-SYP41 antibodies (1:200; Bassham et al., 2000) and anti-SYP21 antibodies (1:200; Conceicao et al., 1997) for primary antibodies and Alexa Fluor® 488-conjugated goat anti-rabbit IgG or Alexa Fluor® 594-conjugated goat anti-rabbit IgG as secondary antibodies.

Fluorescent signal detection and documentation was performed using a confocal laser scanning microscope (Leica TCS/NT, Leica Microsystems, Exton, PA, USA). The confocal laser microscope utilizes a Krypton 568nm and Argon 488nm laser for excitation. Filters for emission were RST588 BP525±25 (FITC-specific detection) and LP590 (TRITC-specific detection). Images were further processed for graphic presentation using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

For all experiments, controls were performed consisting of omission of both primary antibodies (to control for nonspecific staining), omission of only one primary antibody (to confirm that no fluorescence bleed-through between filters was visible in double labeling), and omission of secondary antibodies or all immunochemicals (to control for fixative-induced autofluorescence). The controls confirmed the absence of nonspecific fluorescence. All experiments were carried out at least three times with cells from independent preparations.

Immunoblot analysis. Arabidopsis plants or suspension cells were homogenized in PBS, 1mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 1,000*g* for 10 min at 4°C to remove cell debris and large organelles. The pellet was discarded and the supernatant was incubated in SDS-reducing sample buffer (Biorad, Hercules, CA) for 5 min at 65°C, and separated by electrophoresis on 10% SDS–PAGE gels. Proteins were electrotransferred to nitrocellulose membranes; blots were blocked with PBS/4% low fat milk powder for at least 1 h and incubated with anti-AtSNX2b antibodies for 15-18 h at 4°C. Signal detection was achieved using peroxidase-conjugated secondary antibodies and chemiluminescence reaction followed by X-ray film exposure. For differential centrifugation, the 1,000*g* supernatant from suspension cells was centrifuged sequentially to produce

12,000*g*, 39,000*g* and 125,000*g* pellets and a 125,000*g* supernatant which were analyzed by immunoblot with AtSNX2b antibodies or GFP antibodies (Invitrogen, Carlsbad, CA).

Extraction of AtSNX2b from membranes. A 1,000*g* supernatant from suspension cells was centrifuged at 125,000*g* to produce a total membrane pellet (P125). The supernatant fraction was discarded and membrane pellets were resuspended in 200 μL of extraction buffer (PBS, 1mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) or extraction buffer containing 0.1M Na_2CO_3, 1M NaCl, 2M urea, or 1% (v/v) Triton X-100, and incubated for 2 h on ice. Insoluble material was pelleted at 125,000*g* and pellets were resuspended in SDS sample buffer. Supernatants were precipitated using TCA, and protein pellets were washed in acetone and resuspended in SDS sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting using AtSNX2b antibodies, or SYP41 antibodies as a control (Bassham et al., 2000).

Suc Gradients. Five-d-old *Arabidopsis* suspension cultures were homogenized in HKE buffer (50 mM Hepes-KOH, pH 7.5, 10 mM potassium acetate, and 1 mM EDTA) containing 400 mM Suc, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, and centrifuged at 1,000*g* for 5 min to generate a postnuclear supernatant. The supernatant was loaded onto a Suc step gradient as described in Sanderfoot et al. (1998). Gradients were centrifuged at 150,000*g* in a swinging-bucket rotor at 4°C for 18 h. Fractions (1 mL) were collected from the top of the gradient. Protein in each fraction was analyzed by SDS-PAGE and immunoblotting. Blots were probed using antibodies against aleurain (1:2000; Ahmed et al., 2000), γTIP (1:500; Hicks et al., 2004), FUM1 (fumarase; 1:500; Behal and Oliver, 1997), SYP21 (1:1000; Sanderfoot et al., 2001b), AtSNX2b (1:1500) and VTI12 (1:500; Bassham et al., 2000) followed by secondary antibodies conjugated to horseradish peroxidase.

FM4-64 staining of protoplasts. Protoplasts were stained with FM4-64 according to Ueda et al. (2001) by incubation for 10 min at 4°C with 50 μM FM4-64 in MS medium containing 0.4M mannitol. They were washed three times with the same medium, followed by incubation at room temperature for 30 min to 8h. Confocal microscopy was performed with a Leica TCS/NT confocal microscope (Leica Microsystems, Exton, PA, USA) as described above.

Viability assays. Protoplasts were incubated in 50µg/mL fluorescein diacetate for 30 minutes followed by visualization under a fluorescence microscopy using a FITC filter. Counts for viable (fluorescent) and nonviable (unstained) cells were performed and recorded. Four replicates of at least 700 cells per treatment were analyzed.

Supplemental Material

Figure S1. Viability of Arabidopsis protoplasts.

Results

Three putative SNXs have been identified in the model plant Arabidopsis (Vanoosthuyse et al., 2003). Here we focus on AtSNX2b, a potential SNX based on amino acid similarity (40-50%) with yeast and human SNXs, with the majority of the similarity residing in the region of the PX domain. Of the Arabidopsis sorting nexins, AtSNX2b is most similar to AtSNX2a (86% amino acid similarity) and shows lower similarity to AtSNX1 (47% amino acid similarity). AtSNX2b has two major domains: (1) an N-terminal conserved PX domain which defines a SNX (Worby and Dixon, 2002; Carlton et al., 2004; Carlton et al., 2005) and (2) a C-terminal coiled-coil region potentially important for protein-protein

interactions (Zhong et al., 2002; Leprince et al., 2003; Merino-Trigo et al., 2004; Carlton et al., 2005; Gallop and McMahon, 2005; Figure 1A).

AtSNX2b is a ubiquitously-expressed membrane-associated protein. To determine the expression pattern of *AtSNX2b*, RNA was extracted from different *Arabidopsis* plant organs and RT-PCR was performed using *AtSNX2b* gene-specific primers. Figure 1B shows that *AtSNX2b* expression can be detected in all of the plant organs tested, including roots, rosette leaves, cauline leaves, stem, flowers and siliques, suggesting that its function is important throughout the plant.

To analyze the AtSNX2b protein, antibodies were raised against recombinant AtSNX2b. Full-length AtSNX2b protein was synthesized in *E. coli* as a His-tagged fusion protein and purified by affinity chromatography. The protein was injected into rabbits and the generated antibodies were affinity-purified against the recombinant protein prior to use. Immunoreactivity of the affinity-purified antibodies (Figure 2A) was compared with that of the crude serum (Figure 2C) and preimmune serum (Figure 2B) by immunoblotting against the recombinant protein antigen and a protein preparation from Arabidopsis. The purified anti-AtSNX2b antibodies recognized the recombinant protein and a band of similar size in a total protein preparation from Arabidopsis (Figure 2A), which was not recognized by the pre-immune serum (Figure 2B). Cross-reacting bands were present in the crude serum blots (Figure 2C) that were mostly absent after affinity purification.

It was observed that in addition to a prominent band of the expected molecular mass, a second, weak band of slightly lower mobility on SDS-PAGE was sometimes recognized by the AtSNX2b antibodies. We hypothesized that this band may either correspond to a modified form of AtSNX2b, or, because of the sequence similarity between AtSNX2a and

AtSNX2b, may correspond to AtSNX2a. To investigate this further, an Arabidopsis knockout mutant was isolated from the GABI-Kat flanking sequence tag database (Rosso et al., 2003) in which the AtSNX2b gene was disrupted by a T-DNA insertion (Figure 2D). Loss of gene expression was confirmed by RT-PCR using gene-specific primers. Comparison of protein extracts from the mutant with those from wild-type plants demonstrated that the major band recognized by the AtSNX2b antibodies was absent in the mutant, confirming that it corresponds to AtSNX2b itself. The higher, much weaker band was still present in the mutant, suggesting that this is most likely AtSNX2a, rather than a modified version of AtSNX2b.

Because SNXs are typically associated with membranes, a total protein extract was separated into membrane and soluble fractions by centrifugation at 125,000*g* and analyzed by immunoblotting using AtSNX2b antibodies. AtSNX2b was detected in both the pellet and soluble fractions, indicating that AtSNX2b is partially membrane-associated (Figure 2A). To analyze further this membrane association, differential centrifugation was performed at 12,000*g*, 39,000*g* and 125,000*g* and fractions were probed for the presence of AtSNX2b. AtSNX2b was detected in all three membrane fractions and the soluble fraction, confirming that AtSNX2b is partially membrane-associated (Figure 2D). The upper weak band, potentially AtSNX2a, also appeared to be membrane-associated, although the weak and variable cross-reactivity made it difficult to draw definitive conclusions about this protein.

To determine how AtSNX2b protein associates with the membrane, total membrane pellets were resuspended in either extraction buffer alone, or extraction buffer containing 2M NaCl, 0.1M Na_2CO_3, 2M urea or 1% (v/v) triton X-100. After incubation for 2 h, membranes were repelleted and pellet and supernatant fractions analyzed by immunoblotting with AtSNX2b antibodies (Figure 2E). Each of the treatments was able to extract AtSNX2b from

the membrane, indicating that AtSNX2b is peripherally associated with membranes. As a control, SYP41, an integral membrane protein (Bassham et al., 2000), was only extracted from the membrane by the detergent triton X-100 (Figure 2E).

AtSNX2b can bind PI3P. SNXs are defined by the presence of a PX domain and its ability to bind phosphoinositol lipids (Worby and Dixon, 2002). To determine if the AtSNX2b PX domain can associate with PI lipids, or phospholipids in general, the PX domain fragment of AtSNX2b was fused with GST (glutathione-S-transferase) to generate GST-PX. As a control, point mutations were introduced into the PX domain to create an amino acid 233RR→LG change that has been shown previously to prevent PI binding in human SNXs (Zhong et al., 2002). GST fusions with the wild-type or mutant PX domains were synthesized in *E.coli*, purified over a glutathione resin (Figure 3A) and allowed to bind to PIP^{TM} strips (Echelon Inc.) containing various phospholipids. Binding was detected using antibodies against GST. The PX domain of AtSNX2b (GST-PX) specifically bound to PI3P while the PX domain mutant (GST-PX-1) and GST alone were not able to bind to any lipid (Figure 3B).

Localization of AtSNX2b. To gain insight into AtSNX2b function, its subcellular localization was examined *in vivo*. A GFP fusion was generated with full length AtSNX2b (GFP-AtSNX2b) and transiently expressed in Arabidopsis protoplasts derived from suspension cultured cells. In addition, immunofluorescence using anti-AtSNX2b antibodies was used to assess AtSNX2b subcellular localization in *Arabidopsis* protoplasts. Both immunofluorescence and GFP-fusion localization show that AtSNX2b localizes to punctate spots in the cytoplasm (Figure 4A and 4B). To verify that a full-length GFP-AtSNX2b fusion is produced and correctly associates with membranes, membrane and soluble fractions were prepared from protoplasts expressing GFP-AtSNX2b, or GFP as a control. The proteins were

expressed for 20 h to allow the proteins to accumulate to high enough levels for detection by immunoblotting using GFP antibodies. A GFP-AtSNX2b fusion of the expected size was detected predominantly in the membrane fraction (Figure 4D).

To analyze the role of the PX domain in localization of AtSNX2b to these structures, a fusion between the full length AtSNX2b protein containing the PX domain mutation described above and GFP was generated (GFP-AtSNX2b-1). In contrast to the wild-type protein, the GFP-AtSNX2b-1 mutant did not localize to discrete structures but rather showed a diffuse fluorescence pattern throughout the cytosol (Figure 4C), suggesting that the PX domain is required for correct localization and membrane association *in vivo*. To determine whether the PX domain alone is sufficient for localization to punctate spots, GFP was fused with the PX domain or mutant PX domain alone and the localization analyzed by fluorescence microscopy. GFP-PX and GFP-PX-1 fusions showed diffuse GFP patterns similar to the GFP-AtSNX2b-1 mutant (Figure 4E). To confirm this result, an additional PX domain mutant was generated (PX-2; 211PP→AA). Unfortunately, the full length GFP-AtSNX2b-2 fusion protein was not expressed in protoplasts, based on GFP fluorescence. The fusion of GFP with the PX-2 mutant PX domain also showed diffuse cytosolic localization, as for the wild-type and PX-1 fusions. Our results suggest that the PX domain is necessary but not sufficient for the association of AtSNX2b with punctate compartments.

As an initial approach to determine the identity of the AtSNX2b-labeled structures, the distribution of AtSNX2b and several known intracellular markers in a Suc density gradient (13-55%) was examined. Fractions from the gradient were analyzed by immunoblotting using antibodies against aleurain (ALEU; vacuolar soluble protein), γTIP (vacuolar membrane protein), fumarase (FUM1; mitochondria), SYP21 (PVC), VTI12 (TGN)

and AtSNX2b. Figure 5 shows that AtSNX2b has a bipartite distribution with part soluble (fractions 2-5) and part membrane-bound (fractions 7-12), as predicted from the differential centrifugation (Figure 2C). The distribution of the membrane-associated portion of AtSNX2b on the Suc gradient overlapped with that of VTI12, suggesting a possible localization to an organelle with similar density to the TGN and distinct from the vacuole and mitochondria.

To determine more precisely the localization of AtSNX2b, roots of transgenic Arabidopsis lines expressing markers for the TGN (T7-SYP42; Bassham et al., 2000), PVC (T7-SYP21 and T7-SYP22; Sanderfoot et al., 1999) and late endosomes (YFP-Rha1; Preuss et al., 2004) were analyzed by double immunofluorescence-labeling using AtSNX2b and T7 antibodies or comparison with YFP fluorescence as appropriate. As we do not have a Golgi marker that is expressed in roots, protoplasts were transiently transformed with the Golgi marker sialyl transferase (ST)-GFP (Wee et al., 1998) and immunolabeled with AtSNX2b antibodies. As in protoplasts, AtSNX2b antibodies recognized punctate structures in root cells that were not labeled with preimmune serum (Figure 6A). To assess the specificity of AtSNX2b labeling, immunofluorescence was also performed under identical conditions on roots from the *Atsnx2b* knockout mutant. No specific signal was seen, indicating that the immunofluorescence staining observed corresponds only to AtSNX2b. White spots in merged images show that some of the AtSNX2b-labeled organelles also contained T7-SYP42, T7-SYP21 (data not shown), T7-SYP22 or YFP-Rha1 (Figure 6B). By contrast, very little overlap was seen between ST-GFP and AtSNX2b, suggesting that AtSNX2b does not reside in the Golgi apparatus.

Percent co-localization was determined by counting the number of AtSNX2b-labeled structures that co-localized with each of the markers. Partial colocalization of AtSNX2b with

T7-SYP42, T7-SYP21, T7-SYP22, and YFP-Rha1 was observed (Figure 6B and C). The PVC and late endosomal markers T7-SYP21, T7-SYP22 and YFP-Rha1 show extensive overlap in their localization (Lee et al., 2004), leading to the conclusion that a large portion of AtSNX2b (over 60%) does not co-localize with any of the markers tested. As our antibodies are specific for AtSNX2b under these experimental conditions (Figure 6A), this localization pattern suggests that AtSNX2b might localize to or cycle between PVC/late endosomal compartments, the TGN, which has been suggested also to be an early endosome (Dettmer et al., 2006; Lam et al., 2007), and possibly an additional unidentified compartment.

Over-expression of AtSNX2b affects trafficking. It is known that Arabidopsis cells contain multiple endosome types (Ueda et al., 2004), and we therefore hypothesized that the unidentified compartment with which AtSNX2b associates could be an additional type of endosome. To test this hypothesis, we analyzed the localization of GFP-AtSNX2b in transiently transformed protoplasts compared with the fluorescent marker FM4-64. FM4-64 is widely used as an endocytic tracer in live cells (Vida and Emr, 1995; Betz et al., 1996; Bolte et al., 2004). The cell cultures used contain cells of varying sizes; no differences were seen between large and small cells in any experiment. FM4-64 binds to the plasma membrane, is internalized by endocytosis, traffics through the endosomal system and reaches the vacuolar membrane after 3-4 h in Arabidopsis (Bolte et al., 2004). Arabidopsis protoplasts were transiently transformed with GFP-AtSNX2b and incubated for 12 h to allow expression of the protein. They were then labeled with FM4-64 and uptake of the dye was observed over a time course of up to 4 h (Figure 7A). After 0.5 h of uptake, FM4-64 staining was seen mainly at the plasma membrane, with a few puncta in the cytoplasm; no colocalization of FM4-64 with GFP-AtSNX2b was seen at this early time point. Between 1 h

and 3 h of uptake, FM4-64 was found in punctate structures corresponding to endosomes (Betz et al., 1996; Bolte et al., 2004). Almost complete colocalization of FM4-64 and GFP-AtSNX2b was seen at these times, confirming that the punctate GFP spots are endosomes, and suggesting that AtSNX2b is predominantly an endosome-localized protein.

By the 4 h time point, FM4-64 reached the vacuolar membrane in control protoplasts transformed with GFP alone, while in GFP-AtSNX2b transformed protoplasts FM4-64 was not present on the vacuolar membrane, instead being trapped in cytoplasmic structures containing GFP-AtSNX2b. FM4-64 was not able to exit the GFP-labeled compartments even after 12 h. In addition, the appearance of the GFP-AtSNX2b structures varied over time. At early time points the GFP-AtSNX2b localized to structures similar in appearance, although apparently somewhat larger than the structures in which endogenous AtSNX2b resides; the size of these organelles is difficult to assess by fluorescence microscopy. At later time points these structures became enlarged and/or aggregated, and by 4 h FM4-64 labeling the protoplasts contained just a few large GFP-AtSNX2b-labeled structures (Figure 7A). This is not related to the presence of FM4-64, as similar effects are seen in the absence of FM4-64 staining, with a gradual increase in the size of the labeled structures over time. The GFP-AtSNX2b in the enlarged structures is most likely membrane-associated, as it pellets with a membrane fraction after lysis of protoplasts (see Figure 4D) and it co-localizes with FM4-64, which is a membrane-bound dye. These results suggest that over-expression of AtSNX2b causes inhibition of FM4-64 trafficking to the vacuole, possibly by blocking the exit of material from endosomes.

To confirm that the inhibition of transport of FM4-64 to the vacuole was caused by an effect on trafficking, rather than a loss of cell viability, protoplasts were transiently

transformed with the GFP-AtSNX2b construct and expression allowed to proceed for up to 24 h. Fluorescence microscopy confirmed the formation of enlarged structures as above, and protoplasts were stained with the vital stain fluorescein diacetate to assay for cell viability. No difference was seen between untransformed and transformed protoplasts, and in both cases almost all protoplasts survived, indicating very little loss of viability (Figure S1).

To verify that over-expression of AtSNX2b alone affects FM4-64 trafficking to the vacuolar membrane, rather than the presence of the GFP tag, an untagged AtSNX2b overexpression construct was introduced into protoplasts, followed by FM4-64 labeling as above. Similar to the effect of GFP-AtSNX2b expression, enlarged FM4-64 structures were observed in the cytoplasm, and most of the FM4-64 failed to reach the vacuolar membrane (Figure 7B), even at later time points. This confirms that over-expression of AtSNX2b affects trafficking along the endocytic pathway to the vacuole.

Biosynthetic protein trafficking to the plant vacuole occurs through at least two major pathways, and markers are available for each pathway consisting of GFP fused to an N-terminal vacuolar sorting signal (NTPP-GFP; Ahmed et al., 2000) or a C-terminal vacuolar sorting signal (GFP-CTPP; Fluckiger et al., 2003; Sanmartin et al., 2007). To determine whether the over-expression of AtSNX2b inhibits either of these biosynthetic pathways in addition to endocytic trafficking, AtSNX2b was co-expressed in protoplasts with either NTPP-GFP or GFP-CTPP. At 15 h after transformation, punctate motile GFP spots were seen (Figure 8A, arrows) along with a clear vacuolar NTPP-GFP or GFP-CTPP signal. By 30 h after transformation, motile GFP spots diminished and vacuolar labeling of GFP became very dominant (Figure 8A). In protoplasts transformed with NTPP-GFP or GFP-CTPP alone, only vacuolar GFP signal was observed after 30 h. In double transformants expressing

NTPP-GFP plus 35S::AtSNX2b, non-motile GFP spots accumulated in addition to vacuolar GFP labeling after 30 h (Figure 8A, arrowheads); in contrast, double transformed GFP-CTPP protoplasts did not accumulate GFP spots and only vacuolar GFP labeling was evident at the 30 h time point. These results indicate that overexpression of AtSNX2b partially interferes with trafficking of NTPP-GFP to the vacuole, whereas no effect is seen on GFP-CTPP trafficking.

To determine whether the structures accumulating NTPP-GFP upon overexpression of AtSNX2b are the same structures in which FM4-64 accumulates, protoplasts overexpressing AtSNX2b and NTPP-GFP were labeled with FM4-64. At 3 h after FM4-64 uptake, FM4-64 labeling co-localized with NTPP-GFP structures that do not reach the vacuole; these colocalized structures persisted up to 12 h after FM4-64 uptake (Figure 8B). This demonstrates that the NTPP-GFP-containing bodies which are the result of over-expression of AtSNX2b are actually endosomes. While most of the NTPP-GFP still reaches the vacuole upon AtSNX2b overexpression, these results suggest that over-expression of AtSNX2b partially interferes with the normal trafficking of NTPP-GFP to the vacuole (Figure 8B).

Endogenous AtSNX2b co-localizes with TGN and endosomal markers (Figures 6 and 7). To determine whether these markers are also present in the enlarged structures produced upon AtSNX2b overexpression, protoplasts overexpressing GFP-AtSNX2b were probed with antibodies against the TGN marker SYP41 (Bassham et al., 2000) or the PVC marker SYP21 (Conceicao et al., 1997) followed by immunofluorescence confocal microscopy. Both SYP41 and SYP21 were present in the enlarged, GFP-AtSNX2b-containing structures and the typical punctate organelles labeled by these antibodies in wild-type cells (Ueda et al., 2004;

Dettmer et al., 2006; Tamura et al., 2007) are largely absent. This indicates that these enlarged structures are likely to be aberrant membrane structures or aggregates containing markers proteins from multiple organelles (Figure 8C and 8D).

Discussion

The sorting nexin family in Arabidopsis. Arabidopsis has three sorting nexins named AtSNX1, AtSNX2a and AtSNX2b (Vanoosthuyse et al., 2003; Jaillais et al., 2006). AtSNX1 is most similar to *Brassica oleracea* SNX1 and yeast Vps5p and has been suggested to function as Vps5p in the plant retromer complex (Jaillais et al., 2007). Vps17p, an additional SNX that partners with Vps5p in the retromer complex, does not have an easily identifiable homolog in *Arabidopsis* but one of the other SNXs may perform this function (Vanoosthuyse et al., 2003; Oliviusson et al., 2006). AtSNX1 functions in the trafficking of the auxin transport component PIN2 through a novel pathway independent from that of PIN1/GNOM (Jaillais et al., 2006). In addition it has been implicated as a component of the retromer complex in *Arabidopsis* (Jaillais et al., 2007; Oliviusson et al., 2006). AtSNX2a and AtSNX2b are highly similar and have been suggested to be duplicate genes with redundant functions (Vanoosthuyse et al., 2003; Jaillais et al., 2006). This study presents the characterization of AtSNX2b as a SNX involved in trafficking in the Arabidopsis endosomal system.

Typical of SNXs, AtSNX2b is present in a soluble and membrane-bound state, with the membrane-bound form most likely to be the active form in trafficking. Consistent with common motifs in SNXs, AtSNX2b has a PX domain in the N-terminal region of the protein and a C-terminal coiled-coil region which is likely to be a BAR domain. BAR domains form

curved structures that can sense membrane curvature (Habermann, 2004; Peter et al., 2004; Ren et al., 2006) and, together with the PX domain, they target the SNX to specific phosphatidylinositol-lipid-rich organelles. The characteristic PX domain of AtSNX2b selectively binds to PI3P *in vitro*, although it is not sufficient to localize GFP to membranes *in vivo*, suggesting that other factors or regions of the protein are also required for membrane association. PI3P lipids are reported in various species to be most abundant in endosomes (Gillooly et al., 2000; Gruenberg, 2003; Lemmon, 2003; Vermeer et al., 2006), Golgi (Gillooly et al., 2001; Vermeer et al., 2006), TGN (Kim et al., 2001), PVC (Vermeer et al., 2006) and the vacuole (Kim et al., 2001; Vermeer et al., 2006). Using a GFP-fused endosomal binding domain in Arabidopsis, Kim et al. (2001) localized PI3P to the TGN, PVC, tonoplast, and vesicles. In addition they proposed PI3Ps to be synthesized at the TGN and transported from the TGN through the PVC to the central vacuole presumably for degradation by vacuolar hydrolases. Consistent with these results, AtSNX2b is localized to endosomes, TGN and the PVC, as determined by colocalization with markers for these compartments and with the fluorescent endocytic marker FM4-64. This localization to multiple compartments suggests that AtSNX2b may cycle between the TGN, PVC and endosomes, and may function in trafficking between or through these organelles.

We have isolated a T-DNA knockout mutant in the *AtSNX2b* gene. Expression of the AtSNX2b mRNA and protein is lost in this mutant, demonstrating that it is a null mutant and is expected to have completely lost AtSNX2b function. Despite this, no obvious phenotype is evident, either at a morphological level or in protein trafficking pathways (data not shown). As the AtSNX2a protein shows a high degree of sequence similarity to AtSNX2b (86% amino acid similarity), and both genes are expressed ubiquitously throughout the plant (see

Figure 1 and www.genevestigator.ethz.ch; Zimmermann et al., 2004) we hypothesize that these two proteins may perform redundant functions. A double mutant in both genes will help to clarify this issue.

Overexpression of AtSNX2b inhibits vesicle trafficking. Overexpression of trafficking components can lead to inhibition of trafficking by disruption of the dynamics of the trafficking process as a result of sequestration of receptors or other factors involved in trafficking (Barr et al., 2000). Expression of high levels of GFP-tagged AtSNX2b leads to the formation of large, GFP-AtSNX2b-containing compartments in the cell. These compartments are most likely enlarged or aggregated endosomes as they accumulate FM4-64, which becomes trapped in these compartments and can no longer reach the vacuolar membrane. They may also contain membrane and cargo derived from multiple sources, as TGN and PVC markers accumulate within them. Overexpression of untagged AtSNX2b gave a similar phenotype in that FM4-64-labeled enlarged compartments are present and FM4-64 in these compartments does not reach the vacuolar membrane. AtSNX2b overexpression also partially inhibited biosynthetic trafficking to the vacuole of the vacuolar marker NTPP-GFP, although at a low efficiency, and most of the NTPP-GFP still reached the vacuole. In a small but consistent number of cells, a portion of the NTPP-GFP was arrested in the enlarged FM4-64-labeled endosomes rather than being transported on to the vacuole. The low efficiency of transport inhibition of biosynthetic cargo compared with endocytic cargo may suggest that inhibition of the biosynthetic transport pathway is a secondary effect of disrupting the structure of the endomembrane system, rather than indicating a direct role for AtSNX2b in vacuolar trafficking.

Based on the localization of AtSNX2b on endosomes, the TGN and PVC, and the overexpression phenotype of enlarged endosomes or endosomal aggregates and inhibition of transport through these endosomes, we hypothesize that AtSNX2b is involved in exit from endosomes. However, there are several possible explanations for the phenotype caused by AtSNX2b overexpression. First, the overexpression of AtSNX2b may interfere with the function of endogenous AtSNX2b. Second, overexpression could interfere with functions of other SNXs by sequestering components common to multiple pathways. In this case, the phenotype observed could be a result of the disruption of several trafficking pathways involving different SNXs. Finally, sequestration of PI3P due to AtSNX2b binding may prevent recognition of PI3P-rich lipid membranes by other proteins. The phenotype would therefore be a result of blocking multiple PI3P-dependent transport pathways. In this study, we have shown that AtSNX2b overexpression disrupts vacuolar protein trafficking through biosynthetic and endocytic pathways, suggesting that the AtSNX2b sorting nexin may be involved in protein trafficking. Additional experiments are now underway to define a more precise function of AtSNX2b in vesicle trafficking pathways.

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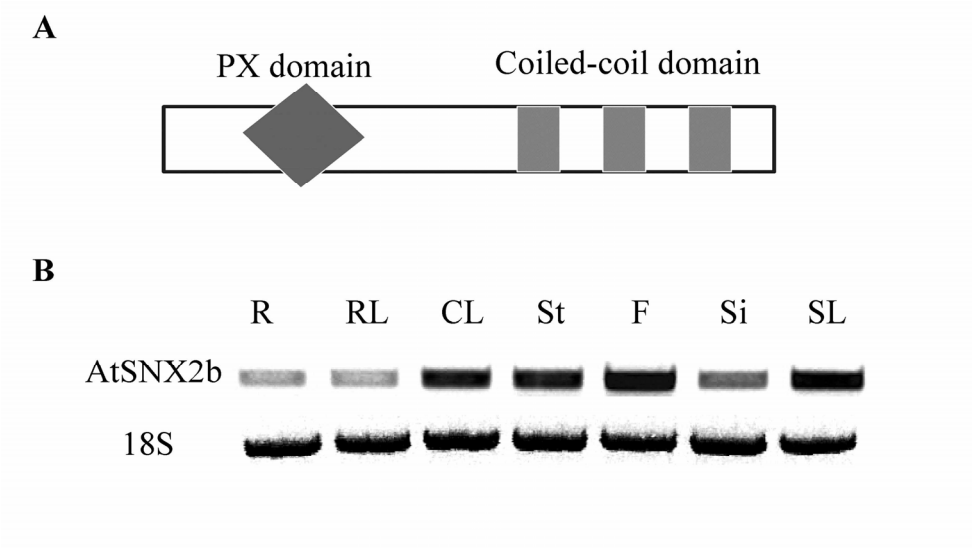


Figure 1. A, Structural features of AtSNX2b. The AtSNX2b protein contains a PX domain near the N-terminus and a C-terminal coiled-coil region. B, Expression of AtSNX2b mRNA throughout the Arabidopsis plant. RT-PCR analysis using AtSNX2b specific primers shows AtSNX2b is ubiquitously expressed in roots (R), rosette leaves (RL), cauline leaves (CL), inflorescence stem (St), flowers (F), siliques (Si) and senescing leaves (SL). 18S RNA is present as a loading control.

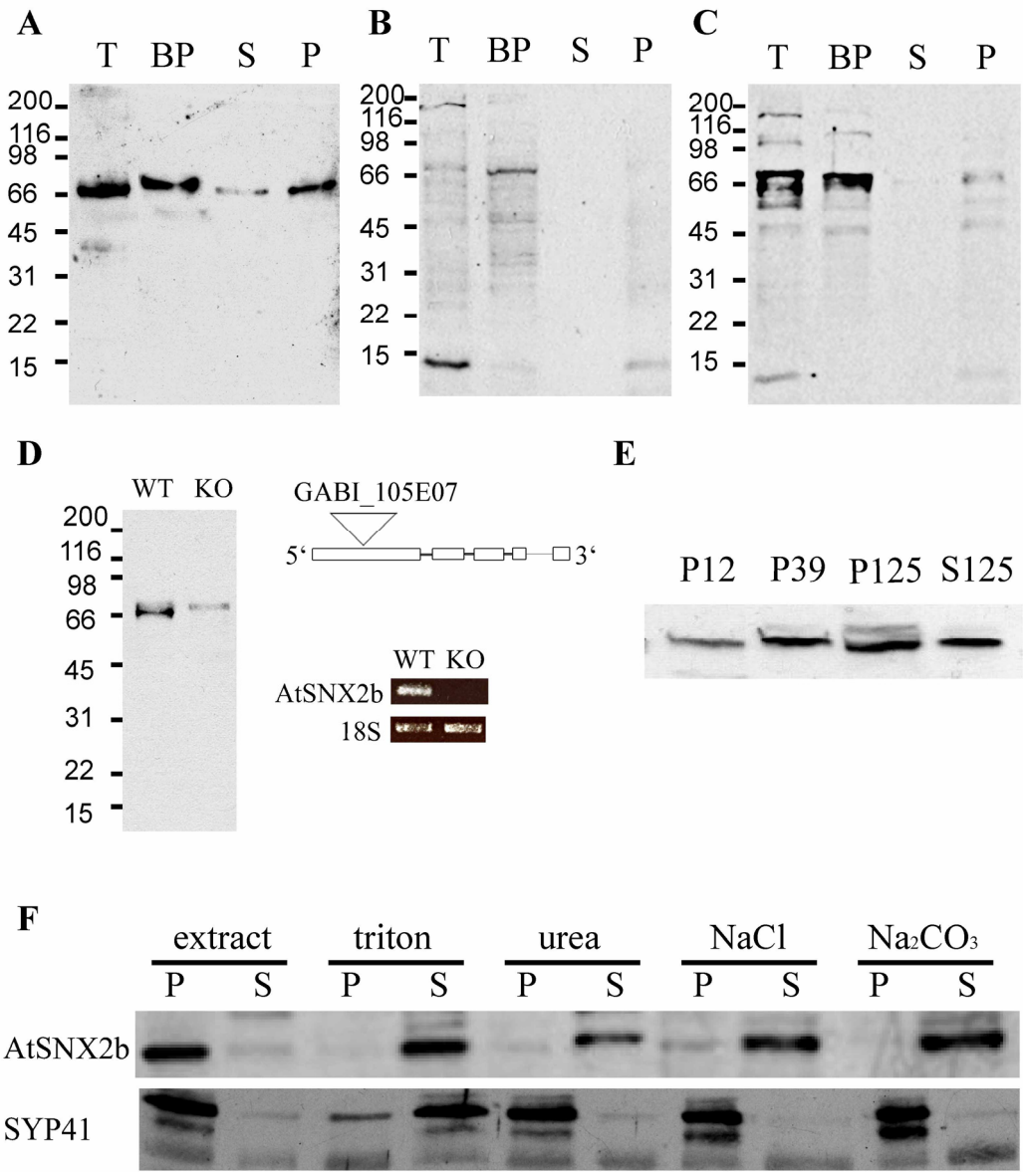


Figure 2. Characterization of AtSNX2b protein in Arabidopsis. Detection of AtSNX2b in protein extracts using A, affinity-purified AtSNX2b antibodies; B, crude immune serum; or C, preimmune serum. Lanes show total Arabidopsis protein (T), *E. coli*-expressed purified protein (BP), total plant soluble (S) and total plant membrane (P) proteins. Molecular mass markers are shown at left (kDa). D, Affinity purified antibodies detect a strong lower and a weak upper band in a total protein extract from wild– type Arabidopsis plants. Only the weak

upper band is present in an extract from the *Atsnx2b* TDNA knockout line GABI_105E07. RT-PCR analysis using AtSNX2b-specific primers indicates that no AtSNX2b RNA can be detected in the GABI_105E07 line. Primers against 18S rRNA are used as a control. E, Differential centrifugation of an Arabidopsis extract to generate 12,000*g* (P12), 39,000*g* (P39) and 125,000*g* (P125) pellets and a 125,000*g* supernatant (S125). Fractions were probed with antibodies against AtSNX2b. F, AtSNX2b membrane association test. Proteins were extracted from a total membrane pellet using extraction buffer alone or containing Na_2CO_3, NaCl, urea, or Triton X-100. Proteins extracted to the soluble phase (S) or remaining in the insoluble pellet (P) were analyzed by immunoblotting using AtSNX2b antibodies, or SYP41 antibodies as a control.

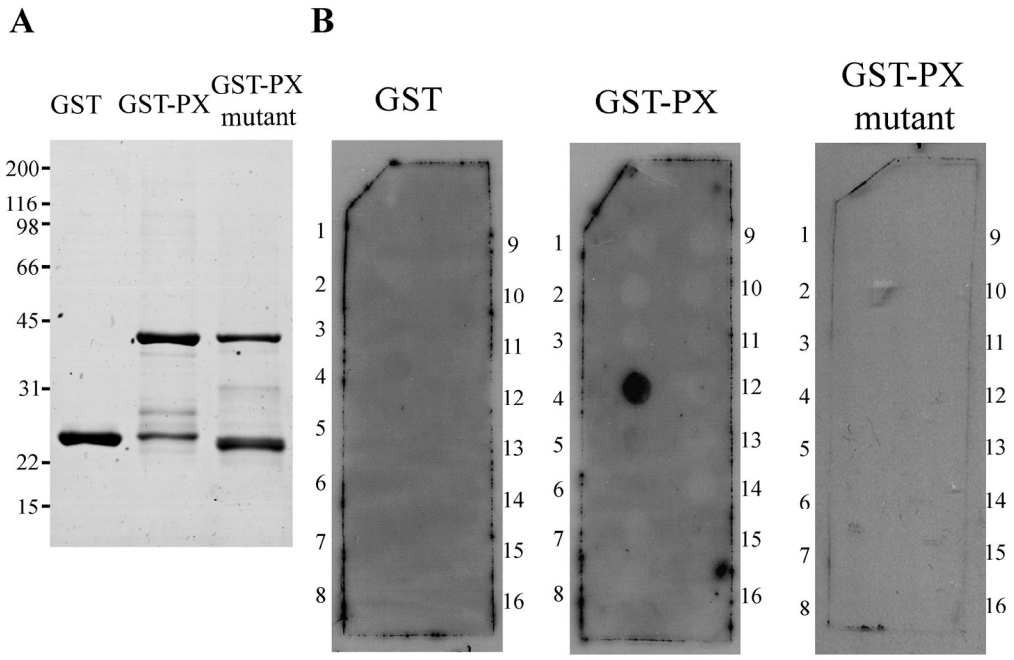


Figure 3. The PX domain of AtSNX2b binds to phospholipids. A, Coomassie-stained gel showing purified GST-fused PX domain and PX-1 mutant, or GST alone as a control, synthesized in *E. coli*. B, Lipid overlay assay for binding to various phospholipids. The purified GST-fusion proteins were allowed to bind to phospholipids immobilized on a membrane (PIP^{TM} strips) and lipid binding was detected using GST antibodies (Invitrogen, Carlsbad, CA). Phospholipids are spotted as follows (1) Lysophosphatidic acid; (2) Lysophosphocholine; (3) Phosphatidylinositol (PtdIns); (4) PtdIns(3)P; (5) PtdIns(4)P; (6) PtdIns(5)P; (7) Phosphatidylethanolamine; (8) Phosphatidylcholine; (9) Sphingosine-1-phosphate; (10) PtdIns(3,4)P; (11) PtdIns(3,5)P; (12) PtdIns(4,5)P; (13) PtdIns(3,4,5)P; (14) Phosphatidic acid; (15) Phosphatidylserine; and (16) no lipid.

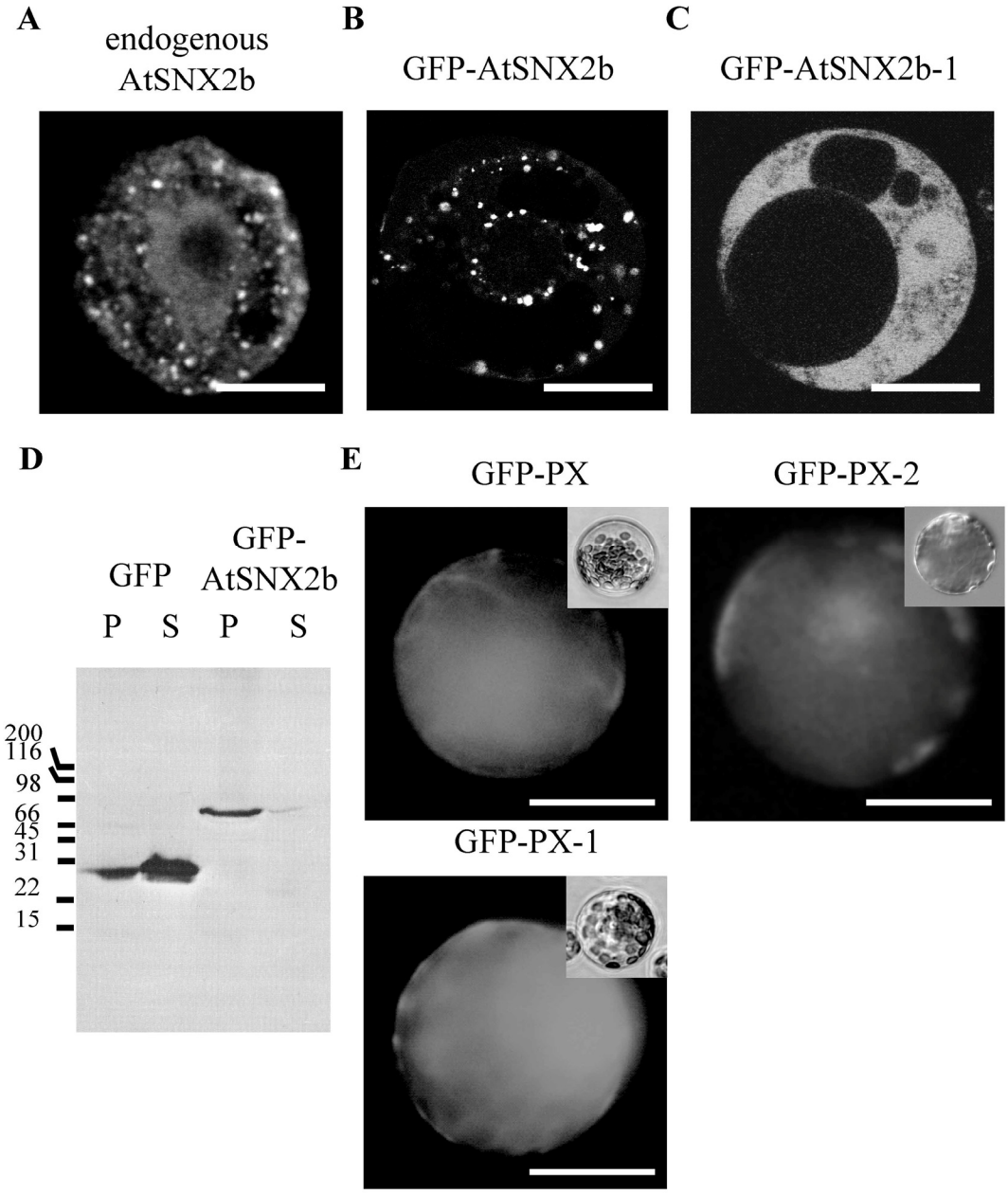


Figure 4. Transient expression of GFP-tagged AtSNX2b and PX domains. A, Immunofluorescence labeling of endogenous AtSNX2b in Arabidopsis protoplasts. B, Expression of GFP-tagged AtSNX2b in Arabidopsis protoplasts. Punctate GFP structures are seen, similar to the endogenous protein labeling in A. C, Expression of the GFP-AtSNX2b-1

PX domain mutant in Arabidopsis protoplasts shows cytoplasmic GFP labeling. D, Immunoblot of GFP-AtSNX2b expression in protoplasts. Extracts from protoplasts transiently expressing GFP-AtSNX2b were fractionated by centrifugation at 125,000*g* yielding soluble (S) and membrane pellet (P) fractions. GFP-fused AtSNX2b is membrane-associated while GFP is mostly soluble. Molecular mass markers are shown at left (kDa). E, Fluorescence images of GFP-tagged PX domain (GFP-PX) and GFP-tagged PX domain mutants (GFP-PX-1 and GFP-PX-2) showing cytoplasmic GFP labeling. Insets show brightfield images. Scale bars for all figures are 10μm.

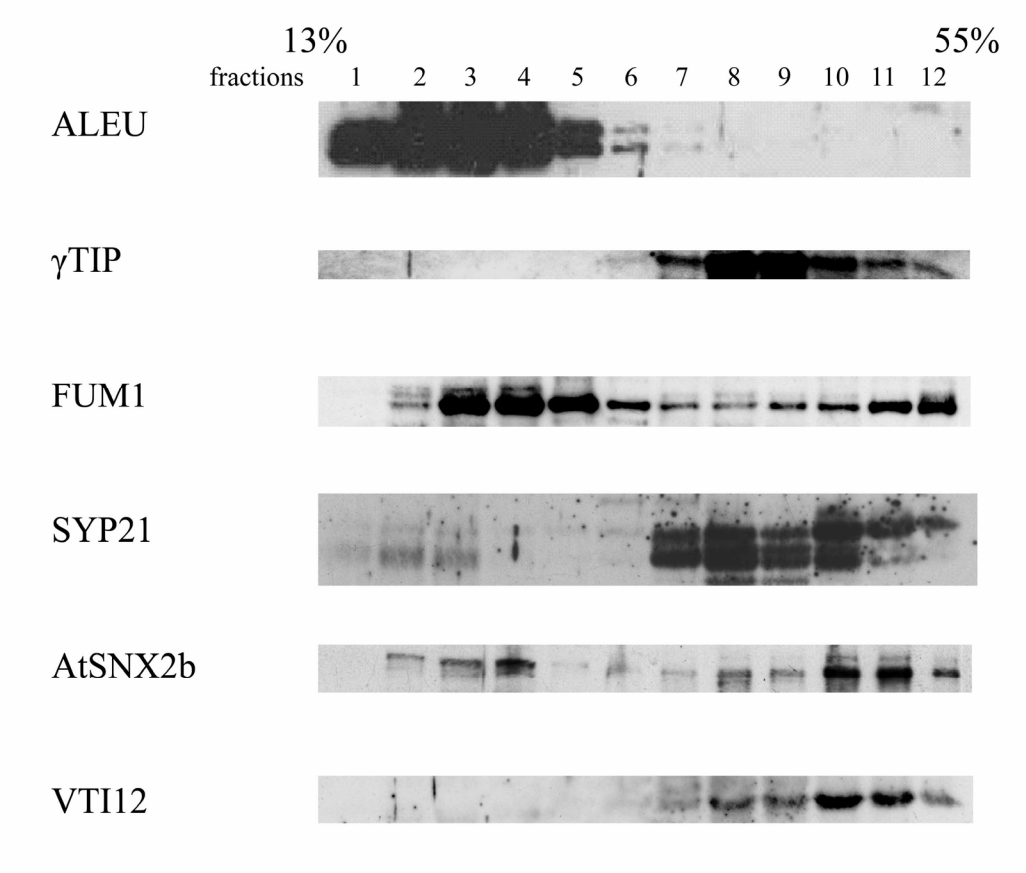
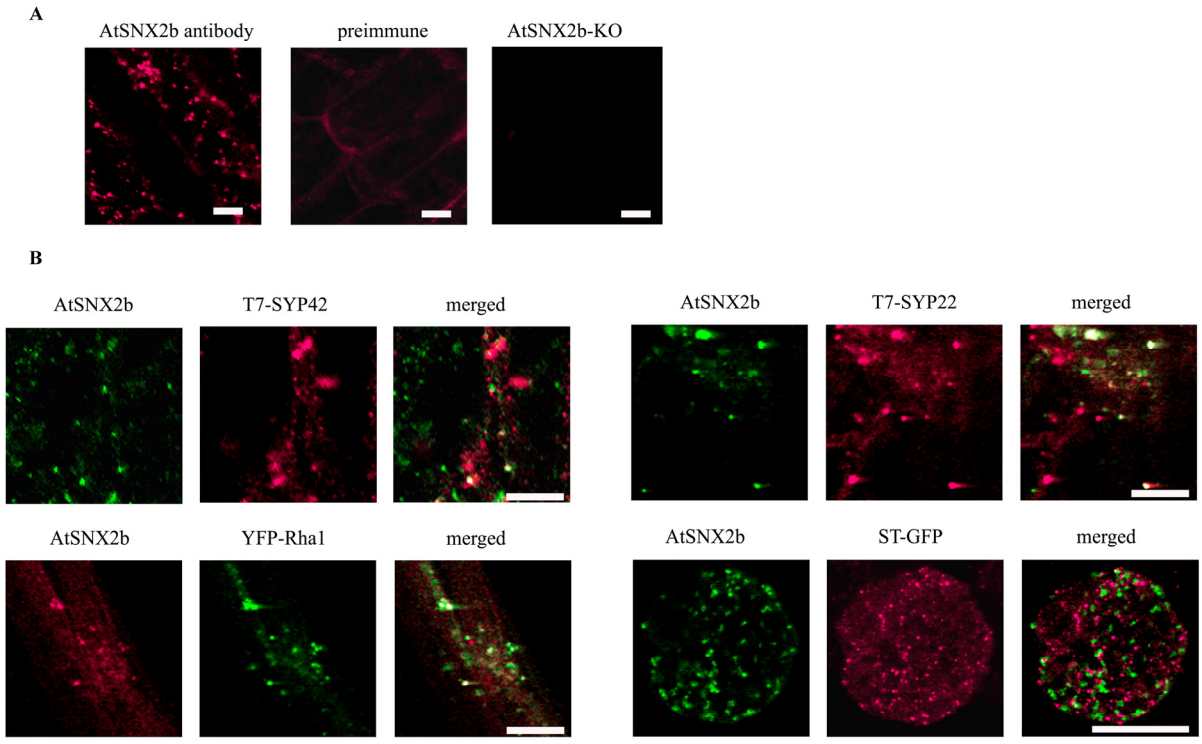


Figure 5. Suc gradient profiles of AtSNX2b and various subcellular markers. An Arabidopsis protein extract was fractionated on a 13% to 55% Suc density gradient and analyzed by immunoblotting with antibodies against aleurain (ALEU), fumarase (FUM1), γ-tonoplast intrinsic protein (γTIP), the t-SNARE SYP21, the v-SNARE VTI12 and AtSNX2b.



C

Markers	Percentage Colocalization
T7-SYP42	11.78±0.57
T7-SYP22	24.41±3.04
T7-SYP21	18.50±0.98
YFP-Rha1	19.35±2.40
ST-GFP	1.30±0.01

Figure 6. Immunofluorescence showing colocalization of AtSNX2b with various subcellular markers in Arabidopsis. Arabidopsis roots were fixed and analyzed by single or double immunofluorescence labeling using T7 and purified AtSNX2b antibodies. A, immunofluorescence using affinity-purified AtSNX2b antibodies detects punctate spots in root cells. Preimmune serum does not produce any specific signal. No signal is seen upon staining with affinity-purified AtSNX2b antibodies under identical conditions in the *Atsnx2b* knockout mutant (AtSNX2b-KO). B, Immunofluorescence images showing double labeling

of AtSNX2b with T7-SYP42, T7-SYP22, YFP-Rha1, and ST-GFP in transgenic plants or protoplasts expressing the appropriate tagged protein. Merged images show partial colocalization of AtSNX2b with each of the tested markers except for ST-GFP. Scale bars represent 10μm. C, Percentage colocalization of AtSNX2b with various subcellular markers.

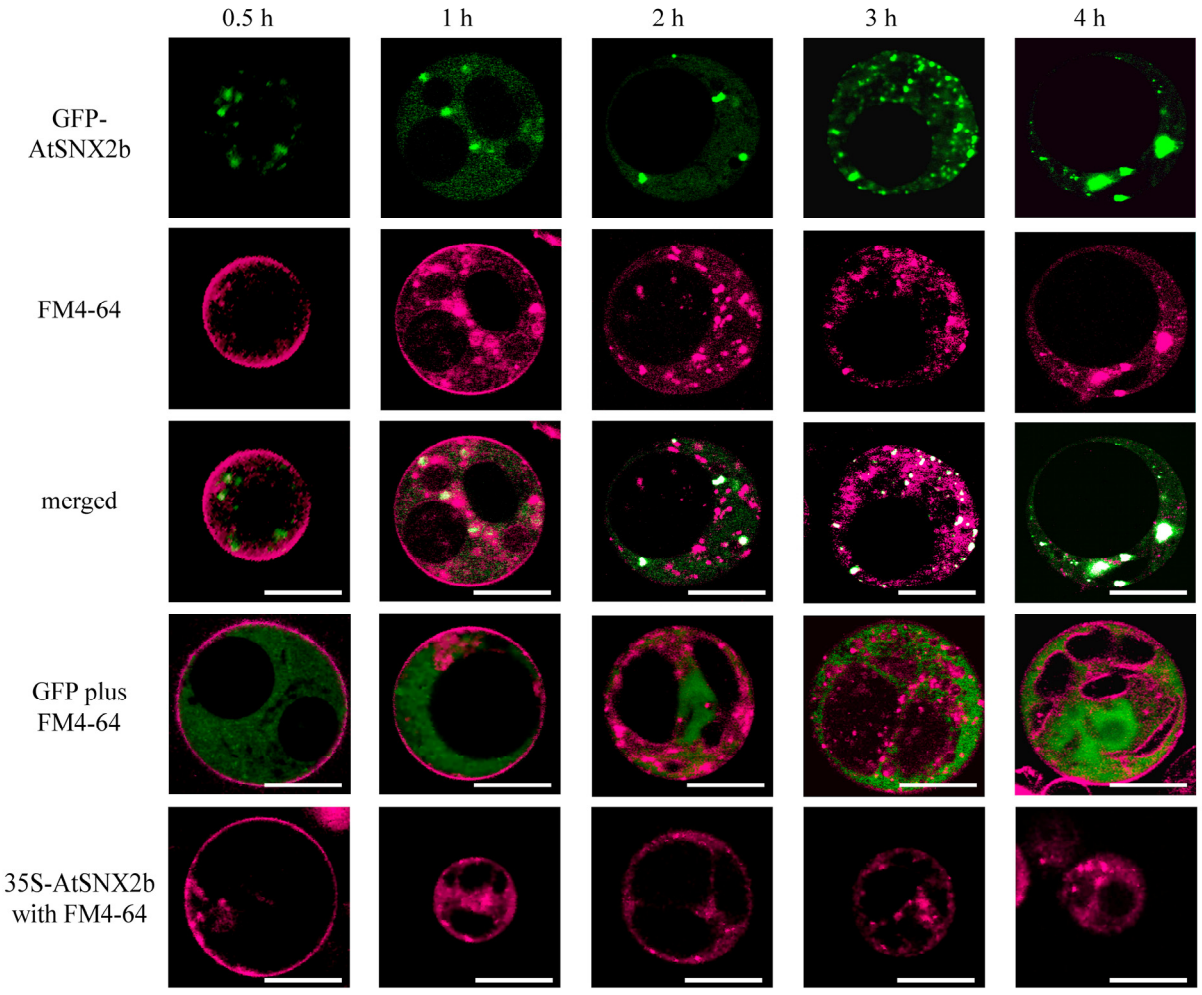


Figure 7. Timecourse of FM4-64 uptake in protoplasts overexpressing AtSNX2b. Arabidopsis protoplasts were transformed with a GFP vector control, GFP-AtSNX2b or 35S::AtSNX2b plus the GFP vector control to tag transformed protoplasts, followed by labeling with the fluorescent dye FM4-64 and incubation for up to 4 h to allow FM4-64 uptake by endocytosis. Scale bars are 10μm.

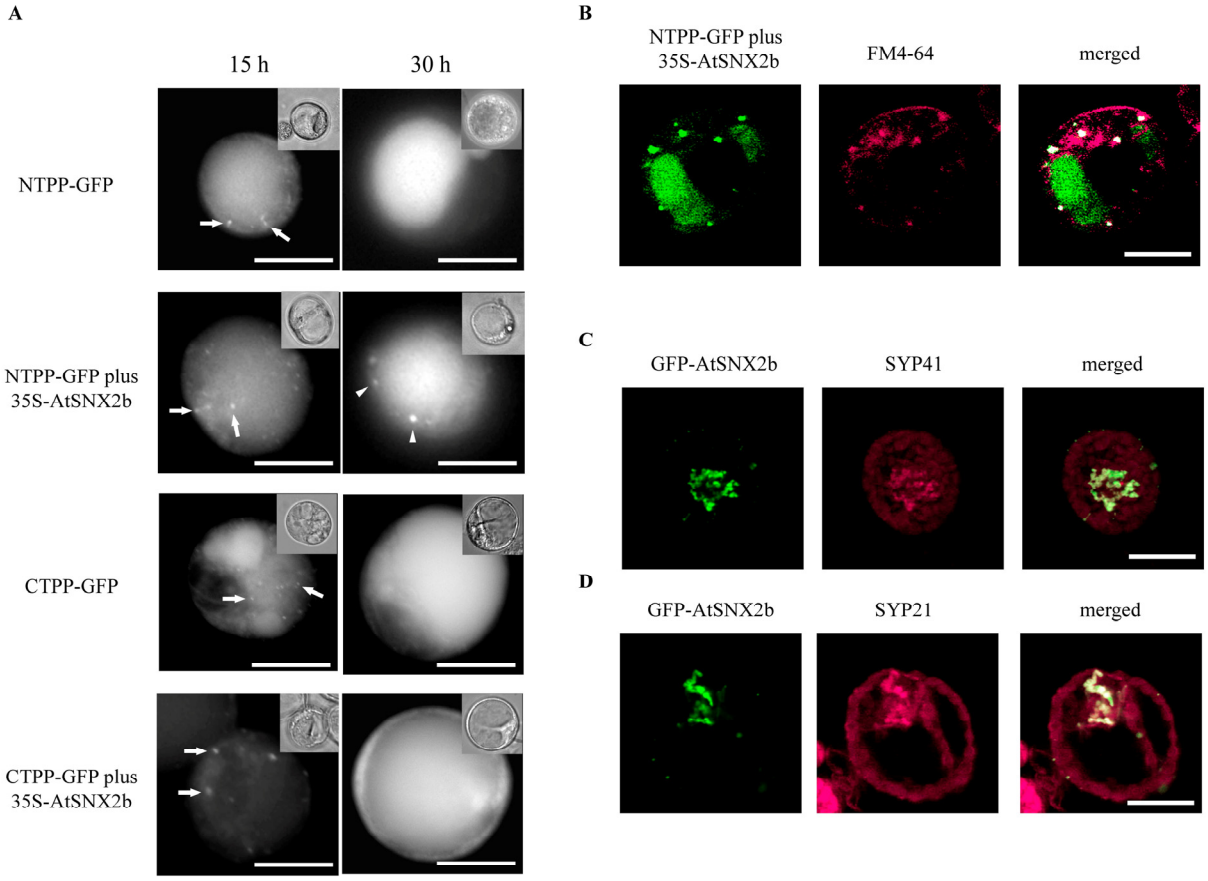


Figure 8. Overexpression of AtSNX2b affects vacuolar trafficking. A, Arabidopsis protoplasts were transfected with the vacuolar markers NTPP-GFP or GFP-CTPP, both +/- 35S::AtSNX2b. At 15 h after transformation, motile GFP spots (arrows) can be seen in all cases and vacuolar labeling of GFP is clear. At 30 h post-transformation, motile GFP spots are absent and vacuolar GFP labeling is more dominant. In protoplasts transfected with NTPP-GFP plus 35S::AtSNX2b, some nonmotile GFP spots accumulate (arrowheads). B, Confocal images of FM4-64 uptake indicate that these GFP-labeled structures are endosomes. C, Immunofluorescence labeling of protoplasts expressing GFP-AtSNX2b with antibodies

against the TGN marker SYP41. D, Immunofluorescence labeling of protoplasts expressing GFP-AtSNX2b with antibodies against the PVC marker SYP21. Scale bars are 10μm.

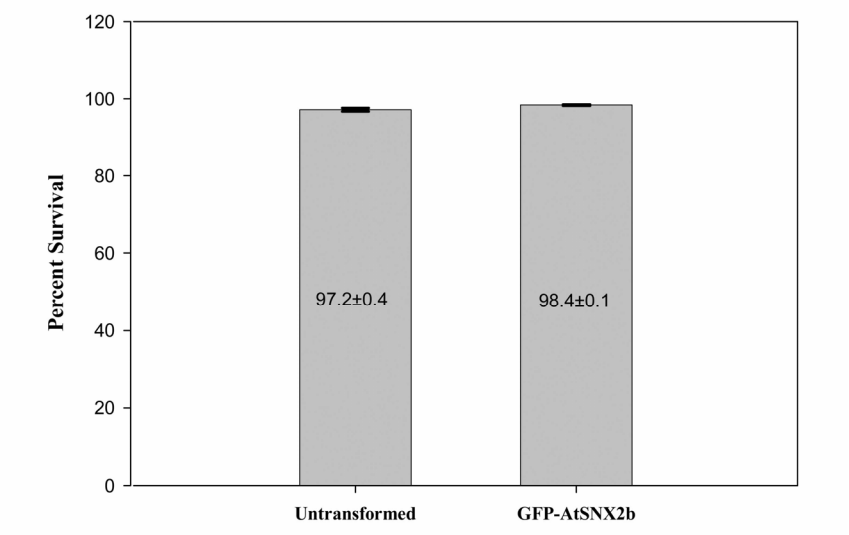


Figure S1. Viability of Arabidopsis protoplasts. Arabidopsis protoplast viability was determined in control (untransformed) and GFP-AtSNX2b-transformed Arabidopsis protoplasts using fluorescein diacetate, 24 h after transformation.

CHAPTER 3. ROOT GROWTH IN ARABIDOPSIS POTENTIALLY INVOLVES SORTING NEXIN MEDIATED-TRAFFICKING

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Abstract

Research on the protein trafficking family of sorting nexins and their role in intracellular trafficking has been increasing in recent years, but only a few studies have determined the contribution of sorting nexins to growth and development of a multicellular organism. Here we examine the Arabidopsis sorting nexin, AtSNX2b and its role *in planta*. Over-expression and null mutants of AtSNX2b show enhanced or reduced root growth, respectively, under nutrient stress conditions during early development of seedlings. *Atsnx2b* KO mutant root growth is slowed by nitrogen-deficient, carbon-deficient and high sucrose stress media. Because the *Atsnx2b* KO can be affected by both carbon-deficiency and carbon abundance, it is possible that the AtSNX2b protein functions in a mechanism that contributes to or regulates root growth. Potential functions of AtSNX2b protein modulation of root growth will be discussed.

Introduction

Organelles in eukaryotes allow them to perform specialized functions such as photosynthesis, lipid metabolism and vacuolar storage. Subcellular compartments (organelles)

like the vacuole, chloroplast and mitochondrion require precise sorting mechanisms to direct proteins to their correct cellular destinations. To maintain these compartments, new proteins have to be synthesized, damaged proteins have to be degraded, while receptor proteins have to be recycled. Although there are different ways to target proteins depending on their final destination, the sorting systems that mediate these processes are crucial to the cell and the overall growth of the organism. Any effects on sorting can cause severe downstream repercussions in growth and development of the whole organism.

Cellular trafficking components are involved in every essential step in targeting, recycling and localizing proteins to their ultimate destinations. The trafficking machinery of the cell can thus function to traffic proteins to their locations. Sorting nexins are one component of the trafficking machinery that bind phosphatidylinositol-rich membranes (Teasdale et al., 2001). These proteins have a PX domain that binds phosphoinositides to localize them to membranes and allows them to act as scaffolds to recruit other proteins to complexes at a particular membrane location (Meijer and Munnik, 2003). The cellular function of sorting nexins is essentially to target proteins that mediate trafficking, recycling or degradation processes; however the physiological function of sorting nexins is not clear. Here we examine the *in planta* function of one sorting nexin, AtSNX2b, in the model plant *Arabidopsis thaliana*. The phenotypic analysis of over-expression and null mutants of AtSNX2b in different stress conditions indicates that AtSNX2b plays an important role in root growth in Arabidopsis. Our data suggest that the protein trafficking molecule AtSNX2b is potentially involved in a mechanism that affects root growth regulation in Arabidopsis. Possible roles of AtSNX2b protein will be discussed.

Materials and Methods

Plant materials and growth conditions. *Arabidopsis thaliana* seeds were surface-sterilized in 33% (v/v) bleach and 0.1% (v/v) Triton X-100 solution for 20 min followed by cold treatment of at least 2 d at 4^{o}C. Plants were grown on soil or AGM medium [Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT), 1% (w/v) Suc (Sigma-Aldrich, St. Louis, MO), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO) and 0.8% (w/v) phytoblend agar (Caisson Lab, Inc., North Logan, UT)] under long-day conditions (16 h light; 100µmol $m^{-2}s^{-1}$) at 22°C.

A homozygous *Atsnx2b* knockout (*Atsnx2b* KO;GABI_105E07) mutant line was received from the GABI-Kat mutant collection at the Max-Planck-Institute for Plant Breeding Research (Rosso et al., 2003). The T-DNA insertion site was verified by GABI_Kat by sequencing the junction between the T-DNA left border and the AtSNX2b first exon. Additional verification of the mutant allele was done by analysis of segregation of the sulfadiazine resistance marker encoded by the T-DNA insertion by growing seedlings in MS medium containing 12 mg/L sulfadiazine (4-amino-*N*-[2-pyrimidinyl]benzene-sulfonamide-Na; for GABI-Kat line).

Growth in nutrient-deprivation media. Seeds were surface-sterilized and cold treated as above and placed on specific nutrient-deprivation medium or nutrient-limiting medium for germination. Nutrient-deprivation media used are as follows: [1] AGM –C (Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT)), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO) and 0.8% (w/v) phytoblend agar); [2] AGM –N (Murashige and Skoog basal salt micronutrient (Sigma-Aldrich, St. Louis, MO), 3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4, 5 mM KCl, 1%

(w/v) Suc (Sigma-Aldrich, St. Louis, MO), 2.4 mM MES pH 5.7 and 0.8% (w/v) phytoblend agar); [3] AGM –N –C (Murashige and Skoog basal salt micronutrient (Sigma-Aldrich, St. Louis, MO), 3 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.25 mM KH_2PO_4, 5 mM KCl, 2.4 mM MES pH 5.7 and 0.8% (w/v) phytoblend agar); [4] AGM –P –S (Murashige and Skoog basal salt micronutrient (Sigma-Aldrich, St. Louis, MO), 3 mM $CaCl_2$, 2.0 mM $NH_4(NO_3)_2$, 3.0 mM KNO_3, 5 mM KCl, 1% (w/v) Suc (Sigma-Aldrich, St. Louis, MO), 2.4 mM MES pH 5.7 and 0.8% (w/v) phytoblend agar).

Nutrient limited media used are the following:[1] ½ MS –C (½ concentration of Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO) and 0.8% (w/v) phytoblend agar); [2] ¼ MS –C (¼ concentration of Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO) and 0.8% (w/v) phytoblend agar); and [3] 1/16 MS –C (1/16 concentration of Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO) and 0.8% (w/v) phytoblend agar).

Root and hypocotyl growth were measured and statistical comparisons were done using ANOVA comparing treatment variables and all interactions generated by the combination of these variables. Analysis was done using the SAS statistical package.

Generation of 35S::AtSNX2b Transgenic Plants. An overexpression line of AtSNX2b (AtSNX2b OX) was constructed using pCAMBIA1300-MCS11 vector (Sanderfoot et al., 2001). A *Bam*HI-*Sal*I fragment consisting of the coding region of the AtSNX2b cDNA (At5g07120) was amplified using primers (forward) 5’-

GGATCCAAAGAAGAGATGGAGAAAC-3' and (reverse) 5'-GTCGACAATTACACTGTGCTCTCATG-3'. The restriction sites are underlined. The *Bam*HI-*Sal*I digested fragment was then subcloned into MCS11 digested with *BglII*-*Sal*I. This construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Mersereau et al., 1990). AtSNX2b OX was introduced into Arabidopsis ecotype Columbia plants by Agrobacterium-mediated transformation using the floral dipping method (Clough and Bent, 1998). Transgenic plants were screened using hygromycin resistance and expression confirmed by RT-PCR and immunoblotting using AtSNX2b antibodies. Homozygous T3 transformant seeds were used for further studies.

Results

AtSNX2b mutant plants. An Arabidopsis AtSNX2b null mutant (*Atsnx2b* KO) was obtained from GABI-Kat, Max-Planck-Institute for Plant Breeding Research (Rosso et al., 2003). Constitutive overexpression lines of AtSNX2b (AtSNX2b OX) were generated by floral dip transformation of wild-type Arabidopsis with 35S-AtSNX2b and progeny were screened for insertion of the T-DNA using antibiotic selection. Expression of AtSNX2b in *Atsnx2b* lines was analyzed by immunoblot of protein extracts from *Atsnx2b* KO and AtSNX2b OX lines. Immunoblot using AtSNX2b antibodies shows that *Atsnx2b* KO lines lack AtSNX2b protein, although a cross-reacting higher band can be detected (Figure 1A). The cross-reacting band detected in the KO is likely AtSNX2a, which has 86% amino acid similarity to AtSNX2b. Immunoblot of extracts from an *Atsnx2b* OX line shows protein levels are several fold higher than in wild-type (WT) (Figure 1B). AtSNX2b OX lines phenotypically resemble WT plants (data not shown), while *Atsnx2b* KO mutants grown on

soil shows varying growth rates compared to WT (Figure 2, top). Because of possible variation in growth that contact to soil can give, *Atsnx2b* KO mutants were grown on Arabidopsis growth medium (AGM) plates to ensure proper contact of roots to medium (Figure 2, bottom). *Atsnx2b* KO plants grown on AGM plates are slightly smaller that WT plants at 2 weeks. At 4 weeks, *Atsnx2b* KO plants can approach the size of WT plants.

Growth of AtSNX2b mutants is affected by carbon and nitrogen availability. To analyze in more detail the growth phenotype, AtSNX2b mutants were grown in varying stress conditions, and the lengths of roots and shoots were compared. Plants were germinated in the dark or light to measure growth (Figure 3). Hypocotyl and root lengths were compared 5 d after germination in the dark (Figure 3A). No significant differences in hypocotyl lengths were seen among plants grown with and without sucrose on AGM (Figure 3B). This suggests that growth of the hypocotyls is unaffected by sucrose in the dark. Significant differences in length were seen in dark-grown roots without sucrose in the medium, with OX seedling root growth rate faster and KO slower than WT (Figure 3B; asterisks). No differences were seen when roots were grown on sucrose-constaining medium in the absence of light. Grown in the light, sucrose in the media enhances growth (Figure 3C). Measurement of root growth in sucrose medium showed that WT and KO lines are not significantly different from each other, but the OX was significantly different from WT (Figure 3C). In no-sucrose media, all lines are significantly different from each other, and the *Atsnx2b* KO root growth rate is the slowest compared to both WT and OX lines (Figure 3B and 3C). AtSNX2b OX root growth is faster than WT when grown in the light (Figure 3C; asterisks). This suggests that root growth of *Atsnx2b* KO plants is affected by the presence of sucrose.

To further examine the root effect in KO plants and OX plants, we determined whether root growth as a result of removal of sucrose would have an additive effect when other macronutrients and micronutrients are limiting. To examine the effect, lowering of macro- and micro- nutrients to ½, ¼ and 1/16 MS in addition to removal of sucrose was used to challenge OX and KO mutant plants. Data from standard 1x MS was used in Figure 3. When grown in the dark (Figure 4B) or in the light (Figure 4C), KO root performance was less than that of WT and there was no additive effect when nutrients were decreased. It is likely that the absence of sucrose alone affects KO mutant root growth (Figure 4B and 4C). OX mutant roots grew faster than WT roots in lower nutrients (Figure 4A, 4B and 4C; asterisks). Plants grown in the light with nutrient-limited media can mimic the natural environment in certain soils. This suggests a gain-of-function in root growth in OX lines.

To verify that carbon availability alone affects KO root growth, we analyzed root growth in AGM media lacking several macronutrients essential for growth. AGM without nitrogen (AGM –N), without nitrogen and carbon (AGM –N –C), and without phosphorus and sulfur (AGM –P –S) were used to test root growth. Table 1 summarizes the results of mutant lines grown in varying nutrient conditions. Lack of phosphorus or sulfur does not affect root growth of the mutants while lack of carbon or nitrogen hinders root growth in KO lines. This suggests that the root growth effect is not solely dependent on a carbon source but also a nitrogen source. The combination of carbon- and nitrogen-deficient media does not have an additive effect on root growth in KO roots.

AtSNX2b mutants in high sucrose. Since lack of carbon and nitrogen affects growth and carbon source seems to be crucial to the performance of KO plants grown in the light (Figure 3C and Table 1), the effect of an over abundance of sucrose (carbon source) on KO root

growth was determined. In normal AGM medium (Figure 5A) there is no significant difference in root growth among mutant lines compared to WT. For growth on 6% sucrose, KO roots show a significantly reduced growth rate compared with the WT and OX lines (Figure 5B); an osmotic control using mannitol showed no significant osmotic effect when using 6% sucrose (Figure 5C). *Atsnx2b* KO root growth is affected by both increased levels (6%) and reduced levels of sucrose (0%) but not standard growth levels (1%). This suggests that AtSNX2b may affect regulation of root growth.

Discussion

The plant is directly connected to its environment through its root system. The plant root plays a major role by acting as a barrier and detection system. The root detects modulations in the plants environment such as touch, gravity, water, nutrients and other stimuli (Legue et al., 1997; Coruzzi and Zhou, 2001; Ivashikina et al., 2001; Massa and Gilroy, 2003; Dat et al., 2004; Eapen et al., 2005; Shin et al., 2005). Detection of environmental cues by the roots allows the plant to respond to stresses which may affect the plant's survival. The plant responds to stresses by modulating growth and metabolism depending on the environmental cue presented to it.

In the case of carbon and nitrogen availability, both carbon and nitrogen pathways coregulate metabolism and development (Coruzzi and Zhou, 2001). In our study, we have identified a protein trafficking component, AtSNX2b, which modulates root growth in situations of different carbon and nitrogen accessibility. Root growth rather than shoot growth is affected by the amount of AtSNX2b (Figure 3A and 3B). Though the mechanism is still not clear, overexpression of AtSNX2b is able to enhance early root growth and

development. Conversely, *Atsnx2b* KO roots perform poorly when grown in carbon-deprived medium and nutrient-limited medium without carbon. Initially, the *Atsnx2b* KO phenotype was attributed to an effect on carbon uptake when compared to growth on standard AGM medium (Figure 3C and Figure 4). Under closer examination of specific nutrient stresses, the stresses of deprivation of carbon, nitrogen or their combination all result in poor root performance in *Atsnx2b* KO plants (Table 1).

Plants use two major sugars as sources, sucrose and glucose. Further verification of the effect of carbon availability was obtained by growing on an over-abundant sucrose concentration (Figure 5B). High sucrose medium was used in the test since high glucose affects signaling and growth (Sheen, 1990; Jang et al., 1997; Xiao et al., 2000). At higher sucrose concentration, it was hypothesized that growth of *Atsnx2b* KO roots would be comparable to growth of WT roots due to high availability and possible compensatory effect of carbon abundance in the medium. Growth on 6% sucrose revealed that the AtSNX2b root growth rate is lower compared to the growth rate of WT roots. No sucrose, high sucrose, and no nitrogen therefore all affect the growth rate of *Atsnx2b* KO roots. As such, the defect in root growth in the *Atsnx2b* KO cannot be attributed to a lack of carbon source. In natural environments, growth rate is directly related to nutrient supply (Clarkson and Hanson, 1980), thus, inhibition of growth is seen in limited and deprived medium and not in nutrient rich or abundant medium. AtSNX2b is therefore probably involved in an indirect mechanism to regulate root growth based on nitrogen or carbon availability. Alternatively, AtSNX2b protein could be a positive regulator of root growth; in normal situations, lack of AtSNX2b does not affect root growth but, in the null mutants, the nutrient stress and lack of AtSNX2b

protein results in a combination of stresses that slows root growth. Following this idea, the overexpression of AtSNX2b positively regulates root growth, and enhances root growth.

Growth studied here is during the germination and early development of the plant and not during later stages of plant development. During later plant development (3-4 weeks), *Atsnx2b* KO plants develop more tertiary root branching than WT and OX plants (unpublished data). The increased root area is hypothesized to compensate for the root defect in the KO. The short time this study employs can only allow conclusions to be drawn from germination and early development affected by AtSNX2b. In the *Atsnx2b* KO plants, the lack of AtSNX2b could either decrease root growth or decrease the ability to regulate this growth. As AtSNX2b is a trafficking-related protein, the phenotype may be a result of a defect in transport of a receptor or nutrient transporter that possibly affects root growth. Because the crosstalk between carbon and nitrogen pathways can complicate further understanding of AtSNX2b function *in planta*, a more detailed analysis of AtSNX2b function is required to better understand its effect in trafficking and/or its effect on root growth. In addition, it is possible that a similar gene in the Arabidopsis genome, AtSNX2a (86% amino acid similarity), could have redundant functions with AtSNX2b. An examination of double knockouts for both AtSNX2a and AtSNX2b is in progress and will shed light on the roles of both sorting nexins in the effect on root growth.

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Table1. Comparison of root growth in varying nutrient conditions. Relative root growth is compared among WT, OX and KO lines grown in the presence of light.

Nutrient Media	KO[§]	WT[§]	OX[§]
AGM	***	***	*****
AGM –C	*	***	*****
AGM –N	*	***	***
AGM –N –C	*	***	***
AGM –P –S	***	***	***
½ MS –C	*	***	***
¼ MS –C	*	***	***
1/16 MS –C	*	***	*****

[§]Asterisks represent relative growth performance of roots. Root performance is compared to WT plants showing control WT growth (***), decreased growth compared to WT (*) and increased root growth compared to WT (*****).

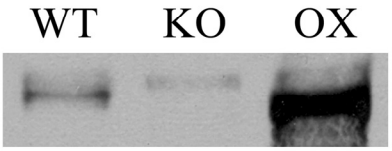

Figure 1. AtSNX2b mutants. Mutant lines were verified for the presence or absence of AtSNX2b protein. Immunoblot of protein extracts from WT, KO and OX lines using AtSNX2b antibodies shows the presence or absence of AtSNX2b proteins.

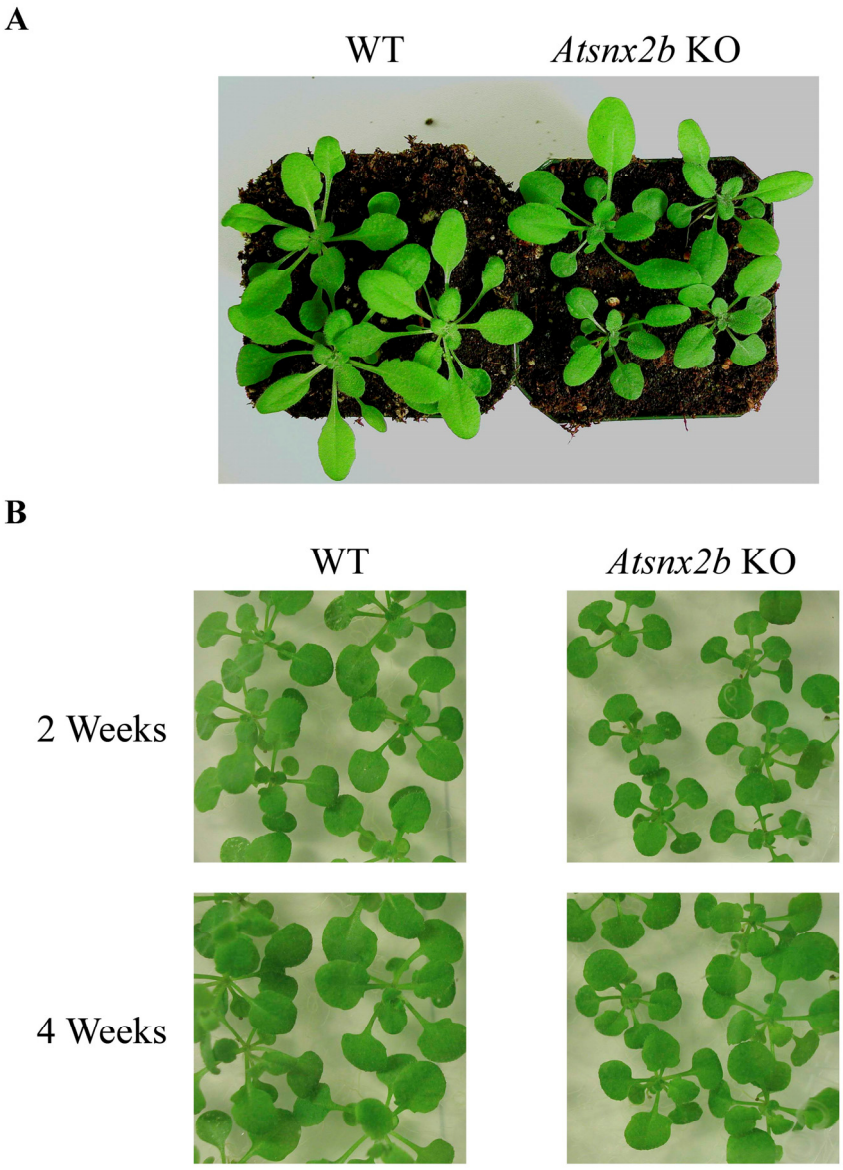

Figure 2. Phenotype of *Atsnx2b* KO mutants. A, Growth of *Atsnx2b* KO plants was compared with WT on soil. B, Mutants were grown on AGM media plates.

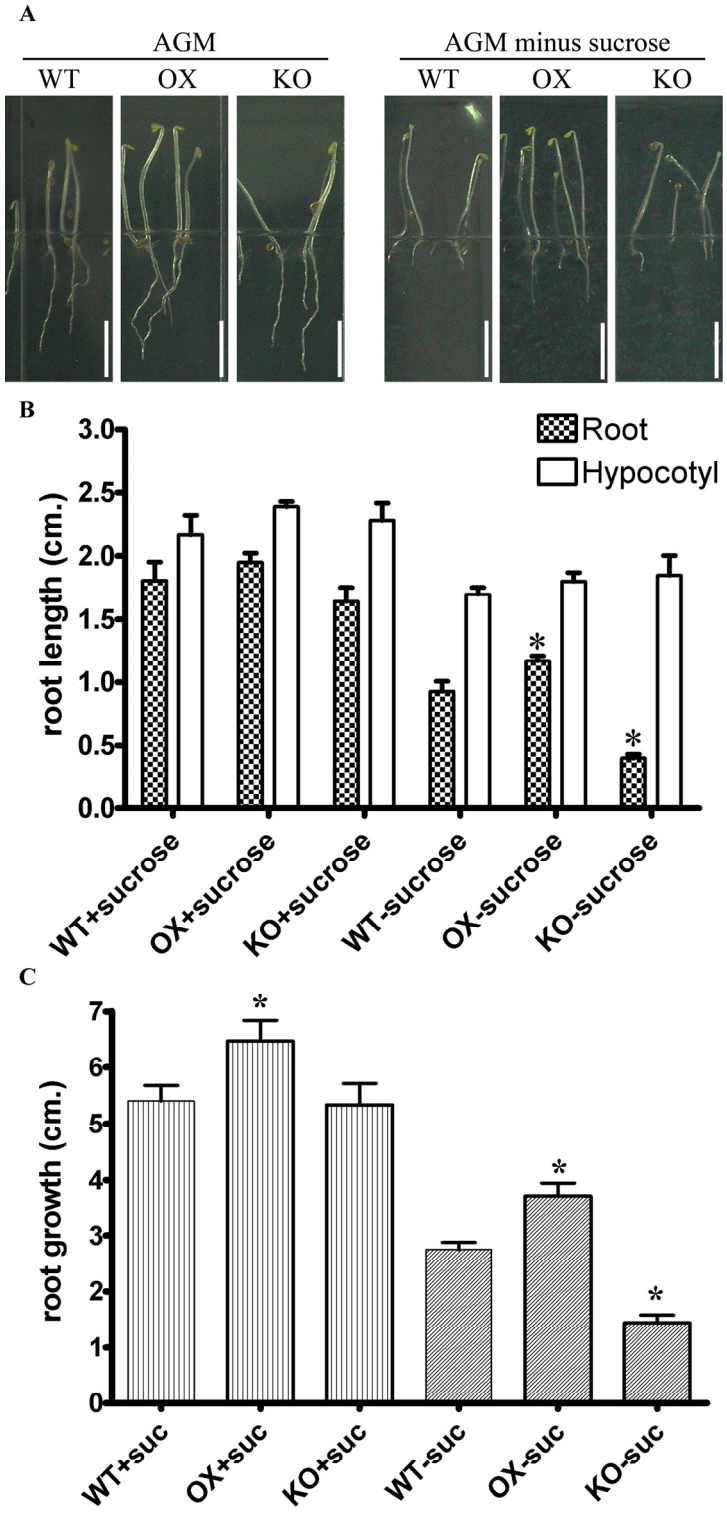

Figure 3. AtSNX2b mutants grown on carbon-limiting media. Plant mutant lines were grown on media with or without sucrose as a carbon source and in the presence or absence of light

for 5 d after germination. A, Phenotype of 5 d-old AtSNX2b mutants grown in the dark. Scale bars are 0.5 cm. B, Quantitative data for hypocotyl and root lengths when grown in the dark. Asterisks show significant differences in plant lines from WT. C, Quantitative data for root length of plants grown in the light 5 d after germination. Asterisks show significant differences from WT lines for both sucrose and sucrose-deprived media.

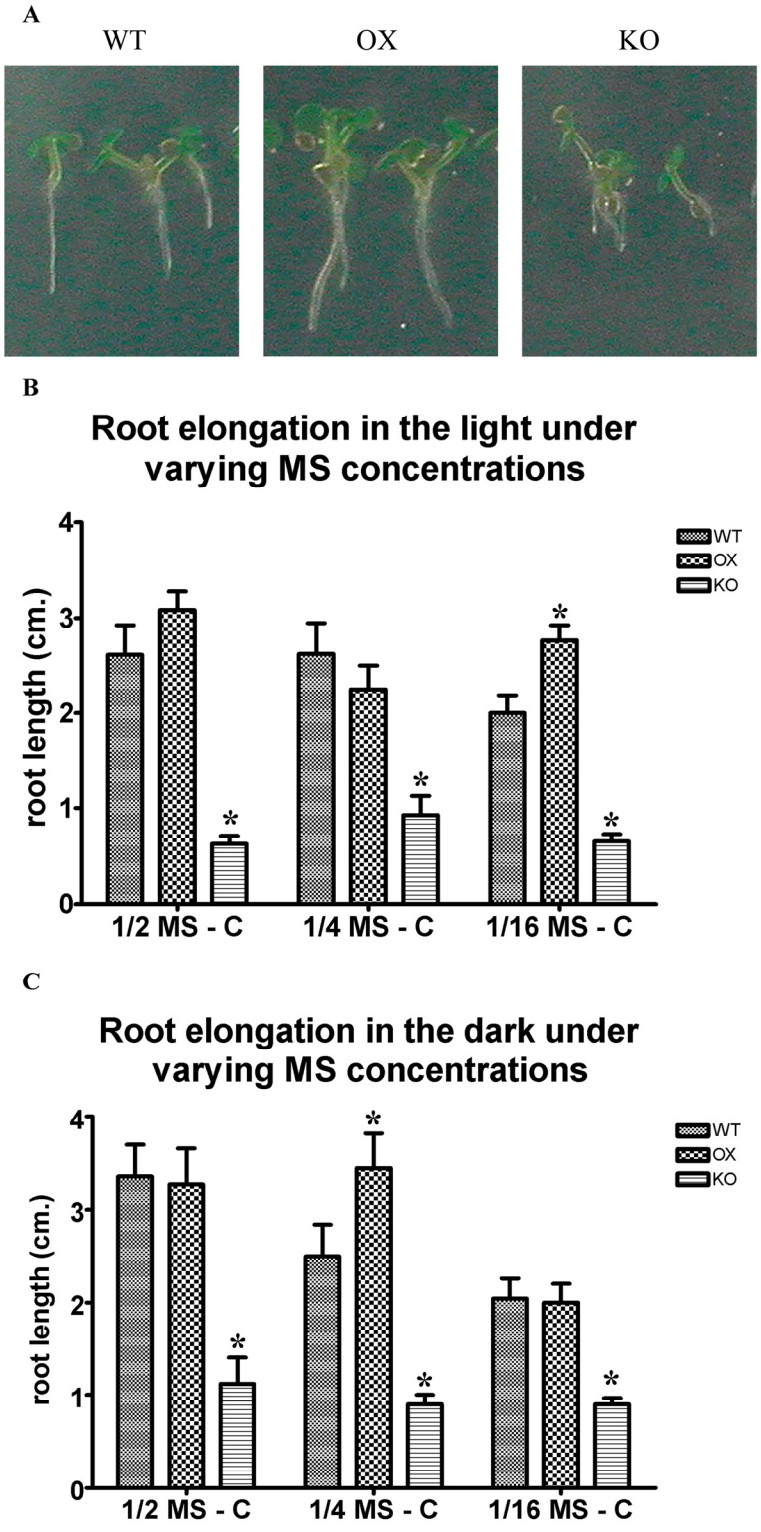

Figure 4. AtSNX2b mutants in low nutrient media. Varying nutrient concentrations without a carbon source were used to test root growth in mutant lines. A, AtSNX2b mutant plants

grown on 1/16 MS with no sucrose. Quantitative data for root growth in reduced MS concentrations are shown. B, Plant lines grown under the light. C, Plant lines grown in the dark. Asterisks show significant differences in plant lines from WT.

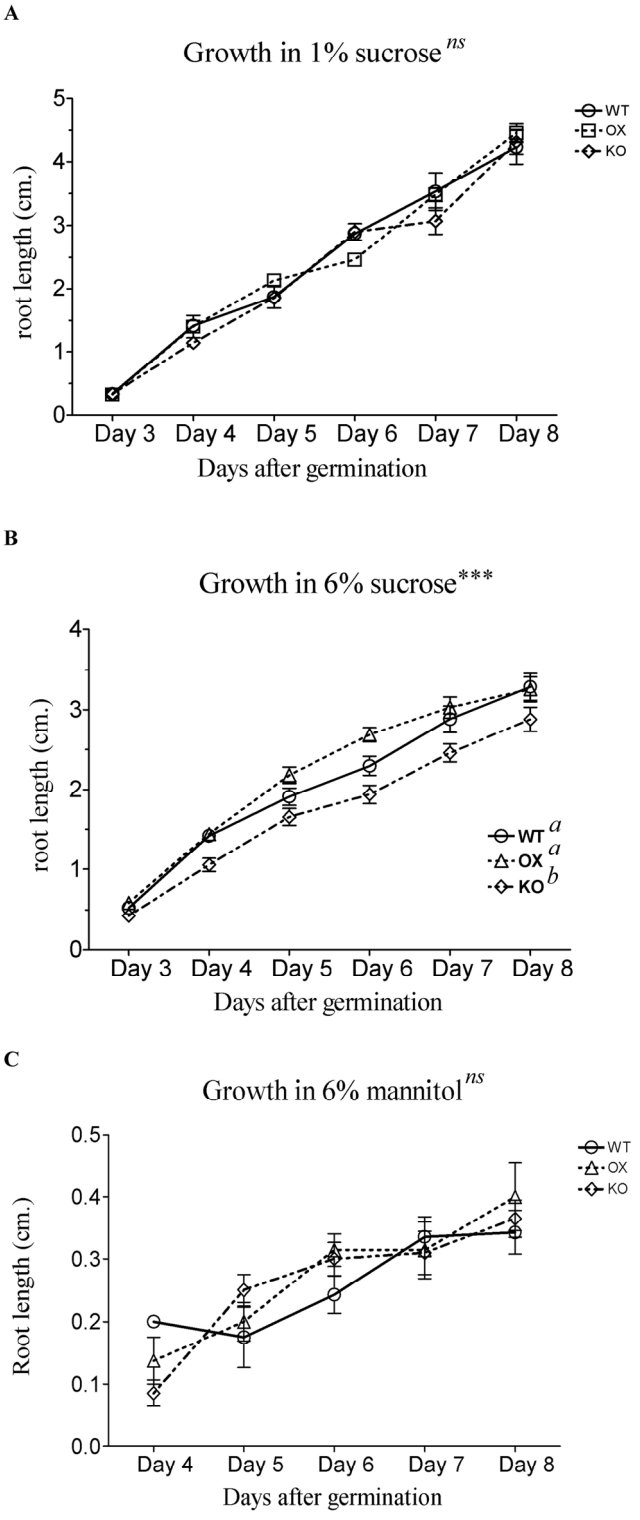

Figure 5. AtSNX2b mutants grown in high sucrose. Mutant lines were grown in standard medium (1% sucrose), 6% sucrose medium and 6% mannitol medium in continuous light.

Growth rate over time was measured among WT, OX and KO lines. A, Growth rate of plants on 1% sucrose medium. B, Growth rate on 6% sucrose medium. C, Growth rate on 6% mannitol, osmotic effect control. Legend: ^{ns} – Growth rates are not significantly different from each other; *** - Growth rates are significantly different from each other; similar italicized letters represent no significant differences between lines.

CHAPTER 4. ANALYSIS OF AtSNX2b-INTERACTING FACTORS

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Abstract

Sorting nexins contain a PX domain and a coiled-coil domain which may be involved in protein-protein interactions. Further characterization of sorting nexin AtSNX2b requires the examination of components that interact with AtSNX2b. Here, we show that AtSNX2b colocalizes with AtSNX1 and with BRI1-GFP. AtSNX2b is localized to two structures, endosomal compartments and the *trans*-Golgi network (TGN). The association of AtSNX2b with BRI1-GFP and with T7-SYP42 was demonstrated. Three new proteins that associate with the AtSNX2b complex were identified but are not predicted to be related to trafficking. These proteins are potential cargoes for the AtSNX2b complex. Potential complexes and their function in relation to AtSNX2b in endosomes and at the TGN are discussed.

Introduction

Endocytic trafficking begins at the plasma membrane, where membrane and extracellular cargo are internalized either by clathrin-coated vesicles or via a clathrin-independent pathway (Baluska et al., 2004; Holstein and Oliviusson, 2005). These vesicles fuse with endosomes followed by further internal trafficking (Muller et al., 2007). Endosome trafficking of receptors, kinases, enzymes and toxins to the *trans*-Golgi network (TGN) in

eukaryotic cells has been demonstrated (Sannerud et al., 2003; Samaj et al., 2005; Bonifacino and Rojas, 2006). Cargo and receptors internalized from the plasma membrane must be destined for a sorting facility in the cell to sort and properly target these proteins to their destination or for degradation. A relatively new branch of thought suggests that the TGN is the essential sorting/re-sorting organelle which directs proteins to and from the plasma membrane and to other membrane locations in the plants (Lam et al., 2007). For the TGN to function in the re-sorting process, various cargos and receptors must be carried from endosomes to the TGN/Golgi.

For vacuolar-targeted cargo, receptors for these cargos are recycled back from the late endosomes to the Golgi for further rounds of sorting. One mechanism of receptor retrieval has been defined by a retrograde trafficking complex now termed the retromer complex (Bonifacino and Rojas, 2006). Sorting nexins (SNXs) are involved in mediating the formation (Schwarz et al., 2002; Bujny et al., 2007; Rojas et al., 2007; Utskarpen et al., 2007) and function (Seaman et al., 1998; Arighi et al., 2004; Seaman, 2004; Seaman, 2005; Jaillais et al., 2006; Oliviusson et al., 2006; Jaillais et al., 2007) of the retromer complex in yeast, plants and mammals. Much effort has been put into the identification of the retromer complex, and increasing evidence has indicated its involvement in protein trafficking and sorting, and association with endosomes (Kurten et al., 2001; Teasdale et al., 2001; Jaillais et al., 2006; Jaillais et al., 2008). To continue to further study the trafficking functions of sorting nexins, the trafficking complexes that associate with these sorting nexins should be determined.

Previous research by Bassham et al. (2000) identified several proteins involved in trafficking from the TGN to the vacuole. Among them, SYP41 and VPS45 were two such

proteins predicted to be involved in vesicle fusion. Identification of additional factors involved in the fusion event employed co-immunoprecipitation with SYP41 antibodies followed by mass spectroscopy techniques (Sanderfoot et al., 2001). One of the identified proteins, sorting nexin AtSNX2b, was selected for further characterization and study. In this study, we examine the endosomal trafficking functions of AtSNX2b sorting nexin through examining the interactions that are formed with the AtSNX2b complex. We demonstrate here that AtSNX2b is co-localized with the endosomal protein AtSNX1 and potentially localizes to BRI1 endosomes. We also provide evidence for possible association of AtSNX2b with the TGN. Other potential interactors in AtSNX2b trafficking were also identified but have yet to be confirmed. These data present potential markers for further study of the role of AtSNX2b in endosomal trafficking in the cell.

Materials and Methods

Plant materials and growth conditions. *Arabidopsis thaliana* seeds were surface-sterilized in 33% (v/v) bleach and 0.1% (v/v) Triton X-100 solution for 20 min followed by cold treatment of at least 2 d at 4^{o}C. Plants were grown on AGM [Murashige–Skoog Vitamin and Salt Mixture (Caisson Lab, Inc., North Logan, UT), 1% (w/v) Suc (Sigma-Aldrich, St. Louis, MO), 2.4 mM 2-morphinolino-ethanesulfonic acid (MES; Sigma-Aldrich, St. Louis, MO), 0.8% (w/v) phytoblend agar (Caisson Lab, Inc., North Logan, UT)] under long-day conditions (16 h light; 100μmol $m^{-2}s^{-1}$) at 22°C. *Arabidopsis thaliana* suspension cell cultures were maintained as described by Contento et al. (2005).

Arabidopsis BRI1-GFP and T7-SYP42 stable line plants for immunoprecipitation were grown in liquid AGM under continuous light with shaking at 120rpm.

Transient transformation of Arabidopsis protoplasts. For suspension cells, protoplasts were prepared and transformed according to Contento et al. (2005). For leaf tissue, protoplasts were prepared and transformed according to Sheen (2002). Protoplasts were transformed with 30µg of DNA per transformation. Images were obtained using fluorescence and confocal laser microscopy.

Immunofluorescence. Three-d-old Arabidopsis seedlings grown on MS solid medium were fixed following Sivaguru et al. (1999). In brief, plants were transferred into 5 mL of MTSB buffer (50 mM PIPES-KOH [pH 6.9], 5 mM EGTA, and 5 mM $MgSO_4$) containing 5% (v/v) dimethyl sulfoxide for 15 min at room temperature. Afterward, they were fixed with 4% (w/v) paraformaldehyde in the above buffer containing 10% (v/v) dimethyl sulfoxide for 60 min at 20°C with the initial 10 min under vacuum. Plants were then washed with MTSB prior to immunostaining. Immunofluorescence staining of treated Arabidopsis seedlings was performed according to Müller et al. (Muller et al., 1998). Treated seedlings were incubated with primary antibodies in a humid chamber for 15-18 h at 4ºC, washed three times for 5 min in MTSB and further incubated for 1–2 h at room temperature with conjugated secondary anti-rabbit or anti-mouse IgG antibodies in 3% (w/v) bovine serum albumin in MTSB. Seedlings were washed five times with MTSB and mounted with a coverslip in 50% (v/v) glycerol in phosphate-buffered saline (PBS, pH 7.4). Antibodies used were anti-AtSNX2b antibodies (1:200; primary) and Alexa Fluor® 594-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA; 1:250; secondary).

For protoplasts, transformed protoplasts were fixed in 4% (w/v) paraformaldehyde in MTSB buffer for 20 min followed by 3 washes with MTSBS (MTSB containing 0.4M sorbitol) prior to immunostaining. Protoplasts were permeabilized using permeabilization

solution (3% (v/v) Triton X-100, 10% (v/v) dimethyl sulfoxide in MTSB) for 20 min. Immunofluorescence staining of fixed protoplasts was performed according to Kang et al. (2001). Cells were washed five times with MTSB and mounted with a coverslip in 50% (v/v) glycerol in PBS. Antibodies used were anti-AtSNX2b antibodies (1:200) with Alexa Fluor® 594-conjugated goat anti-rabbit IgG as secondary antibodies.

Fluorescent signal detection and documentation was performed using a confocal laser scanning microscope (Leica TCS/NT, Leica Microsystems, Exton, PA, USA). The confocal laser microscope utilizes a Krypton 568nm and Argon 488nm laser for excitation. Filters for emission were RST588 BP525±25 (FITC-specific detection) and LP590 (TRITC-specific detection). Images were further processed for graphic presentation using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA).

Immunoblot analysis. Samples were incubated in SDS reducing sample buffer (Biorad, Hercules, CA) for 5 min at 65°C, and separated by electrophoresis on 10% SDS–PAGE gels. Proteins were electrotransferred to nitrocellulose membranes; blots were blocked with PBS/4% (w/v) low fat milk powder for at least 1 h and incubated with anti-AtSNX2b, GFP monoclonal antibodies (Roche Applied Science, Indianapolis, IN, USA) or T7 monoclonal antibodies (Invitrogen, Carlsbad, CA) for 15-18 h at 4°C. Signal detection was achieved using peroxidase-conjugated secondary antibodies and chemiluminescence reaction followed by X-ray film exposure.

Immunoprecipitation. Arabidopsis suspension cells, BRI1-GFP or T7-SYP42-expressing transgenic plants were homogenized in Lysis Buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, and 0.1mM phenylmethylsulphonyl fluoride) then centrifuged at 1000*g* for 15 min. The supernatant was

incubated for 1-2 hours at 4°C and centrifuged at 25,000*g* for 1 h. The supernatant was subsequently filtered through a 0.22μm filter prior to passing through a binding column. Binding column resin was generated by crosslinking AtSNX2b antibodies, monoclonal GFP (Roche Applied Science, Indianapolis, IN, USA) or T7 monoclonal antibodies (Invitrogen, Carlsbad, CA) to NHS-activated Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ, USA) following the manufacturers instructions. The column was rinsed with 5 volumes of Lysis Buffer then washed with 5 volumes of Wash Buffer (50 mM Tris-HCl, pH 7.5, 0.25 M NaCl, 1mM EDTA, 0.1% (v/v) Nonident P-40, 0.05% (w/v) deoxycholate, 0.1mM phenylmethylsulphonyl fluoride). Proteins were eluted with 3 volume of 0.2mM Glycine pH 2.2 followed by concentration with Amicon concentrators (Millipore, Billerica, MA, USA).

Concentrated proteins were then either probed with specific antibodies by immunoblot or proteins were identified from Coomassie stained bands of interest using MALDI-TOF mass spectrometry by the Proteomic Facility at Iowa State University (Carver Co-Lab, Iowa State University). In brief, trypsinized samples were ionized on a 2,5-dihydroxybenzoic acid matrix followed by mass spectrometry analysis using a ABI Q-Star XL quadrapole-TOF tandem mass spectrometer. *De novo* protein identification was done by peptide mass fingerprinting compared to protein profiles generated from the Arabidopsis genome.

Results

AtSNX2b in endosomes. The structure of AtSNX2b consists of an N-terminal PX domain and a C-terminal coiled-coil domain. Coiled-coils are domains that are involved in protein-protein interactions. This suggests that AtSNX2b may form complexes with other proteins in

its function in trafficking. Sorting nexins can form complexes with other sorting nexins and/or with other trafficking components (Seaman et al., 1998; Worby and Dixon, 2002; Carlton et al., 2005). To test whether AtSNX2b can potentially function with other known sorting nexins, we analyzed whether AtSNX2b colocalizes with the other Arabidopsis SNXs, AtSNX1 and AtSNX2a. GFP-fusions with AtSNX1 and AtSNX2a were generated but the AtSNX2a GFP-fusion did not express when transformed into protoplasts. AtSNX1-GFP was found to partially colocalize with native AtSNX2b in Arabidopsis protoplasts (Figure 1). This suggests that one function of AtSNX2b may be through association with a second sorting nexin AtSNX1. To determine if AtSNX1 can associate with AtSNX2b, immunoprecipitations using GFP antibodies were carried out on AtSNX1-GFP expressing protoplasts. The immunoprecipitation of AtSNX1-GFP from expressing cells was successful, but AtSNX2b could not be detected by immunoblot.

We have shown previously that GFP-AtSNX2b fusions localize to endosomes labeled by FM4-64. The role of AtSNX2b in endocytic trafficking from the plasma membrane was examined. The internalization of BRI1-GFP was used as a marker for receptor endocytosis (Russinova et al., 2004; Geldner et al., 2007; Jaillais et al., 2008). BRI1 is a receptor kinase for brassinosteroid; when brassinosteroid binds the brassinosteroid receptor, phosphorylated BRI1 binds BAK1/SERK3 and increased internalization into the cell to endosomes is observed. In addition, ligand-independent internalization of BRI1 occurs at a low rate (Nam and Li, 2002; Russinova et al., 2004). Arabidopsis roots stably expressing BRI1-GFP were used for immunofluorescence to determine whether AtSNX2b colocalizes with BRI1-GFP. Figure 2A and 2B show partial colocalization of AtSNX2b with BRI1-GFP in the root hair and in the root in internal structures. This suggests AtSNX2b may be involved in BRI1-GFP

internalization. To determine whether AtSNX2b can associate with BRI1-GFP, immunoprecipitations using GFP antibodies were carried out on BRI1-GFP transgenic plants. Immunoprecipitation of BRI1-GFP from transgenic plants was able to pulldown BRI1-GFP (Figure 3; ~130 KDa; left). An AtSNX2b signal can also be detected from BRI1-GFP immunoprecipitations using AtSNX2b antibodies on immunoblots (Figure 3; right). The size of AtSNX2b detected is slightly smaller than in total protein controls, and this can be attributed to some degradation during protein purification and elution from the column. This suggests that AtSNX2b associates with BRI1 and this association may be in endosomes.

AtSNX2b at the *trans*-Golgi Network. We have previously shown that AtSNX2b colocalizes with the TGN marker, SYP42. To test whether AtSNX2b associates with SYP42 at the TGN, immunoprecipitation of T7-SYP42 was done and the co-precipitation of AtSNX2b assessed. Immunoprecipitation using T7 antibodies was carried out on T7-SYP42 expressing plants (Figure 4). Immunoblots of the immunoprecipitates were probed with T7 antibodies and T7-SYP42 (~40KDa) and other higher molecular weight bands were detected. These bands are possibly stable complexes/aggregates of T7-SYP42 or modified T7-SYP42 (Figure 4, left). AtSNX2b was detected in T7-SYP42 immunoprecipitate lanes. The bands detected by AtSNX2b antibodies were smaller than those in total protein lanes, and this was attributed to degradation during protein purification and elution. These results provide evidence that AtSNX2b interacts with T7-SYP42.

Other Factors Interacting with AtSNX2b. Based on current data, AtSNX2b is likely to function in endosomal trafficking and also associates with the TGN. To further examine the function of AtSNX2b we attempted to identify proteins interacting with AtSNX2b in the trafficking process. AtSNX2b antibodies were used to immunoprecipitate from Arabidopsis

suspension cells AtSNX2b and proteins that are in the same physical complex. Proteins that were specifically retained on the antibody column were eluted and concentrated. Proteins were separated on SDS-PAGE gels and stained with Coomassie blue stain to compare proteins precipitated with AtSNX2b antibodies against its preimmune control. After comparison, specific bands present in immune immunoprecipitations and not in preimmune immunoprecipitations were selected for identification. Protein samples were digested with trypsin prior to analysis by MALDI/TOF mass spectrometry (MS). The immunoprecipitation of the AtSNX2b complex identified 3 unknown strong protein bands (Figure 5; named Unknown 1, 2 and 3). These bands may be degradation products of the true proteins due to their size (between 6-15KDa) and since we have seen degradation products in other immunoprecipitations (Figure 3 and 4). *De novo* protein identification by peptide mass fingerprinting from full MS spectra identified the proteins to be a plant intermediate filament (IF)-like protein, an endonuclease and unknown proteins (Table 1). These proteins are not expected to be direct trafficking components and may be potential cargos. Further verification of these proteins as AtSNX2b cargos or as part of the trafficking machinery is needed to understand better their role in relation to AtSNX2b.

Discussion

Sorting nexins are known to form homo-dimers and hetero-dimers with other sorting nexins. The colocalization of AtSNX1 with AtSNX2b suggests that AtSNX1-AtSNX2b may form a sorting nexin hetero-dimer combination. This potential association is hypothesized to be in endosomes, and the location in endosomes is supported by evidence of AtSNX1 in sorting endosomes (Jaillais et al., 2008) and AtSNX2b colocalization with endocytic tracer

FM4-64. Examination of the AtSNX1 and AtSNX2b association by co-immunoprecipitation suggests that the two SNXs possibly do not form a dimer. It may be that AtSNX2a associates with AtSNX2b to form a dimer. The analysis of the potential AtSNX2a-AtSNX2b dimer should be done to assess its contributing role to trafficking and in endosomes.

With AtSNX2b interacting with endosomes containing BRI1-GFP receptor kinase, more evidence is presented supporting the role of plant sorting nexins in endosomal trafficking. A similar association of a sorting nexin with a plant receptor kinase has been shown previously through a yeast two-hybrid assay by Vanoosthuyse et al. (2003). BoSNX1 interacts with the kinase domain of receptor-like kinases which interact with SERK kinase family members (Vanoosthuyse et al., 2003). Similarly, the interaction of BoSNX1 and SERK kinase can resemble that of Arabidopsis AtSNX2b indirectly associated with BAK1/SERK3 by the BRI1/BAK1 complex. The BRI1/BAK1 complex is internalized into endosomes, and it is possible that this complex includes AtSNX2b. This complex could have multiple functions such as desensitization of the receptor kinase, degradation of receptor and further signaling by the receptor. This result raises the possibility that endosomal signaling of the BRI1 receptor complex (Geldner et al., 2007) may involve at least one sorting nexin, AtSNX2b. A more detailed examination of AtSNX2b and BRI1 internalization/trafficking/signaling will allow better understanding of how endosomal signaling is affected by a protein trafficking component such as AtSNX2b. This study would give us a better understanding of the roles of sorting nexins in plant growth and development.

The association of AtSNX2b with the TGN SNARE SYP42 has been shown here. Although the function of AtSNX2b protein at the TGN is not clear, the possibility that vesicle trafficking to or from the TGN may require AtSNX2b exists. Because of the

possibility of different dimers of AtSNX2b, specific dimer may function in TGN-related trafficking and another dimer in endosome-related transport. Further examination of AtSNX2b at the TGN is needed to address its role at this organelle.

Several unknown proteins were identified by association with AtSNX2b. From their identity, these proteins are not predicted to be trafficking components and are possibly cargos in vesicles or endosomes containing AtSNX2b. Localization of these cargos to the endosomes or the TGN would provide further evidence of their roles with AtSNX2b; this association with AtSNX2b needs to be confirmed and their localization tested. A detailed analysis of AtSNX2b association with these cargo proteins will address the role of AtSNX2b in the trafficking of each of the potential cargo protein. Much work is needed before the function of AtSNX2b can be truly understood. The combined examination of AtSNX2b involvement in BRI1-containing endosomes, at the TGN and with the identified potential cargo proteins will broaden the understanding of how AtSNX2b functions in the cell.

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Table 1. Summary of identification of unknown proteins.

	Predicted Protein	Protein type	Description
Unknown 1	At3g05830	alpha-helical IF (intermediate filament)-like protein	Ntp1 domain – Archaeal/ vacuolar-type H^+-ATPase subunit I
Unknown 2	At2g48100	exonuclease	ExoIII domain – exonuclease
Unknown 3 (mixture)	At3g19370	unknown protein	DUF869 domain. Family consists of a number of sequences found in *Arabidopsis thaliana*, *Oryza sativa* and *Lycopersicon esculentum*
	At1g50830	unknown protein	DUF1723 domain. The members of this family are expressed in various plant species, and include aminotransferase-like proteins, putative mutator-like transposases and hypothetical proteins.

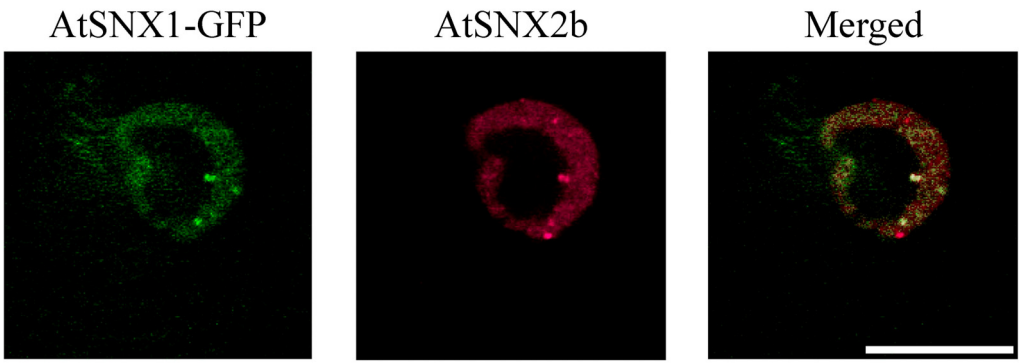

Figure 1. AtSNX2b colocalization with AtSNX1-GFP. AtSNX1-GFP expressed in Arabidopsis protoplasts colocalized with native AtSNX2b protein detected by immunofluorescence using AtSNX2b antibodies. Scale bar is 10μm.

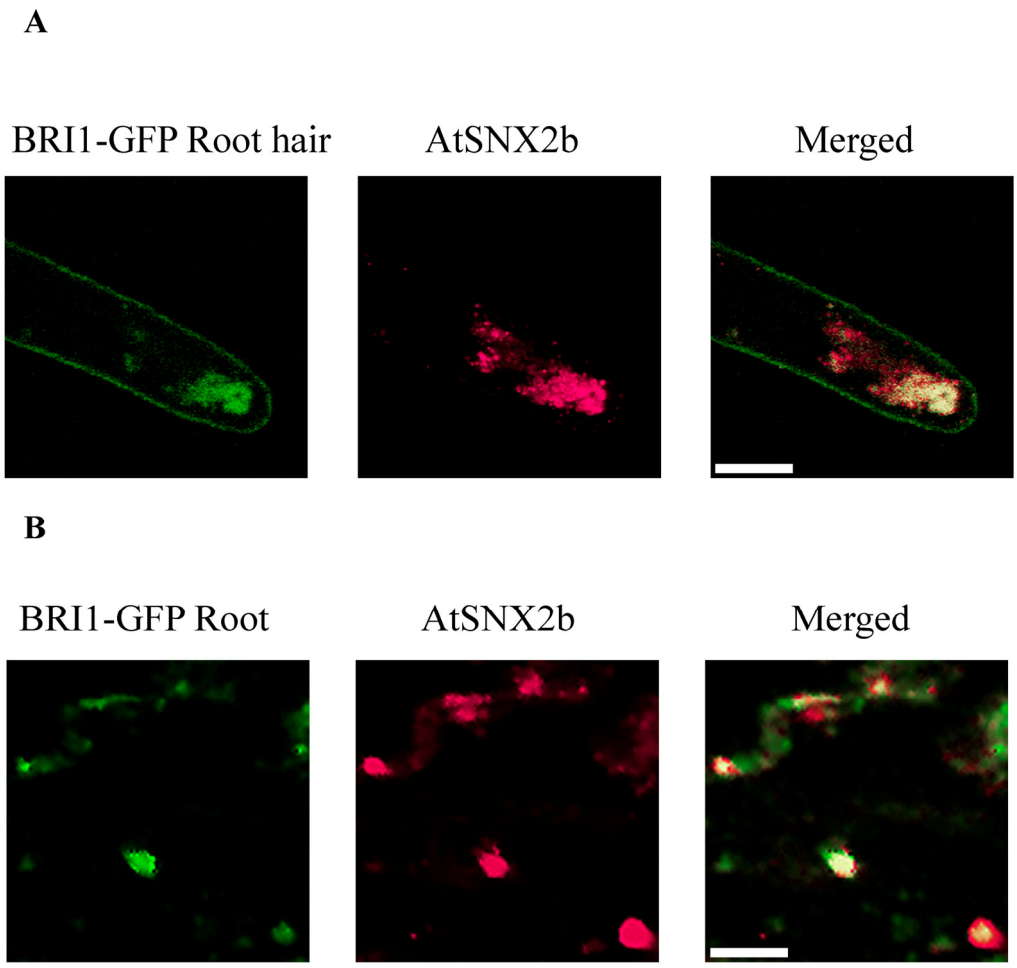

Figure 2. BRI1-GFP and AtSNX2b localization. Immunofluorescence was performed on fixed roots of BRI1-GFP transgenic plants using AtSNX2b antibodies. BRI1-GFP signal in the root is low while BRI1-GFP is more abundant in root hairs. Images show that BRI1-GFP and AtSNX2b partially colocalize in BRI1-GFP root hairs (A) and in roots (B). Scale bars are 10 μm.

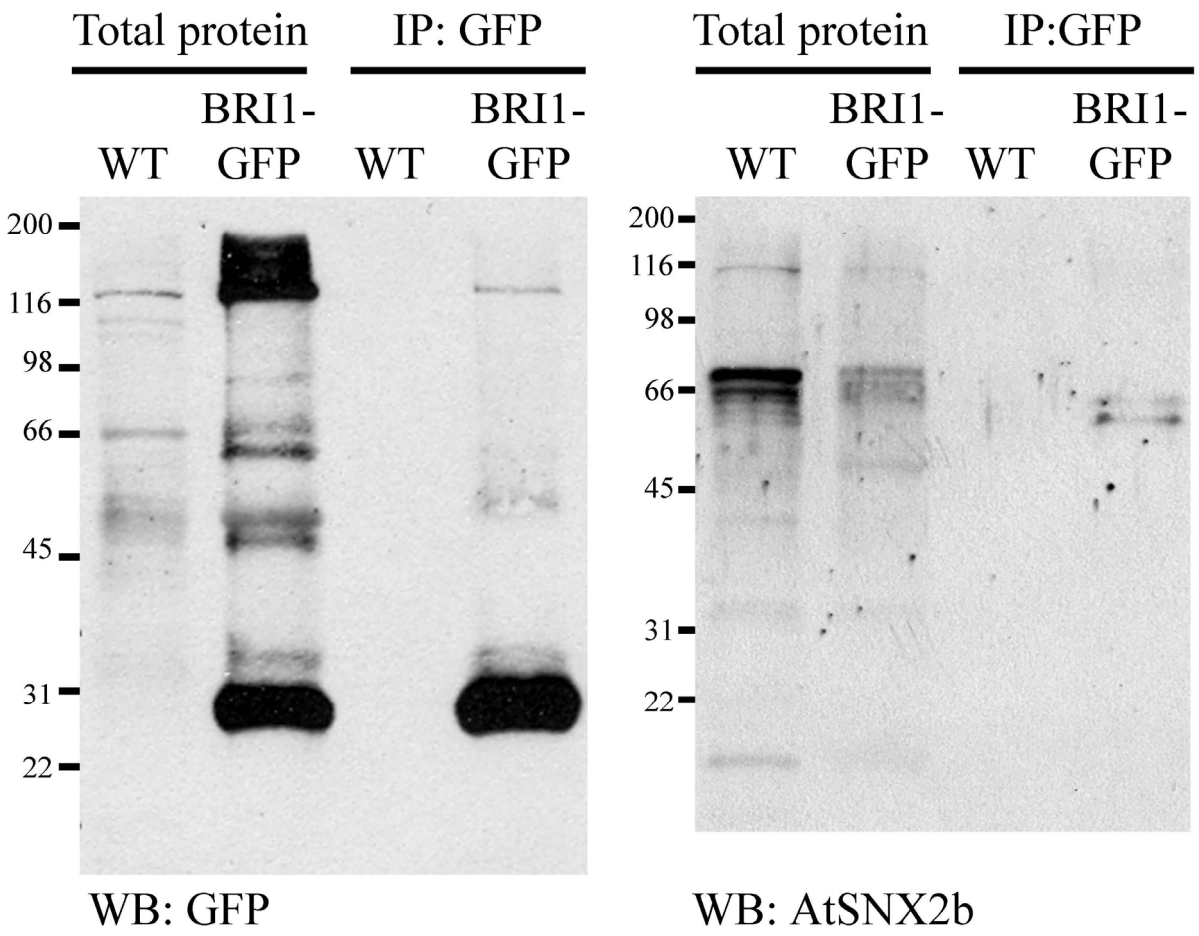

Figure 3. Co-immunoprecipitation of AtSNX2b with BRI1-GFP. BRI1-GFP was immunoprecipitated using a GFP antibody column. The protein complexes were eluted from the column and concentrated followed by immunoblot analysis. *On the left*, western blot (WB) probed with GFP antibodies showing detection of GFP-tagged BRI1. Left two lanes are total proteins for the WT and BRI1-GFP samples. Right two lanes are GFP immunoprecipitated samples for WT and BRI1-GFP. *On the right*, western blot probed with AtSNX2b. Left two lanes are total protein control for WT and BRI1-GFP samples. Right two lanes are GFP immunoprecipitates for WT and BRI1-GFP.

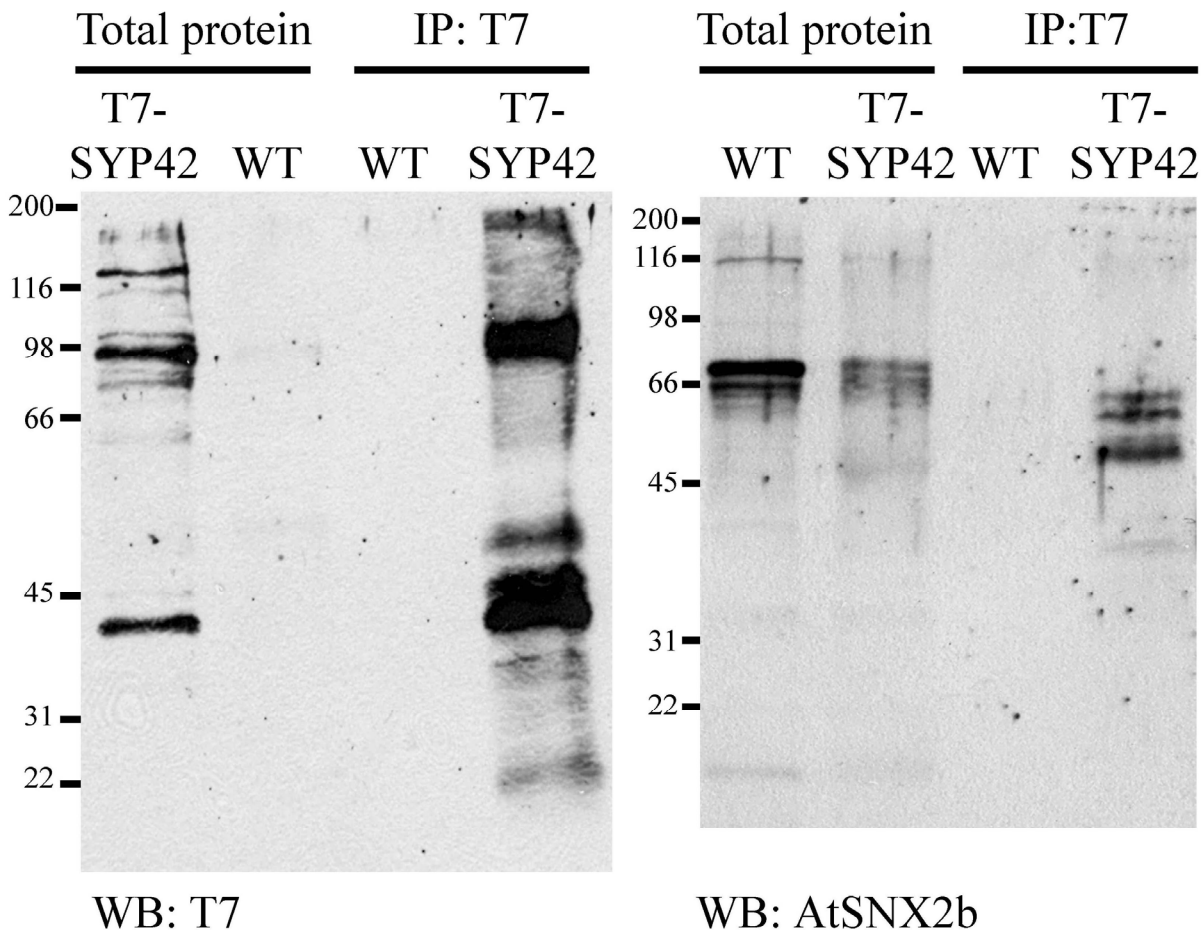

Figure 4. Co-immunoprecipitation of AtSNX2b with T7-SYP42. T7-SYP42 was immunoprecipitated using a T7 antibody column. The protein complexes were eluted from the column and concentrated followed by immunoblot analysis. *On the left*, western blot (WB) probed with T7 antibodies showing detection of T7-SYP42. Left two lanes are total proteins for the T7-SYP42 and WT samples. Right two lanes are T7 immunoprecipitated samples for WT and T7-SYP42. *On the right*, western blot probed with AtSNX2b. Left two lanes are total protein control for WT and T7-SYP42 samples. Right two lanes are GFP immunoprecipitates for WT and T7-SYP42.

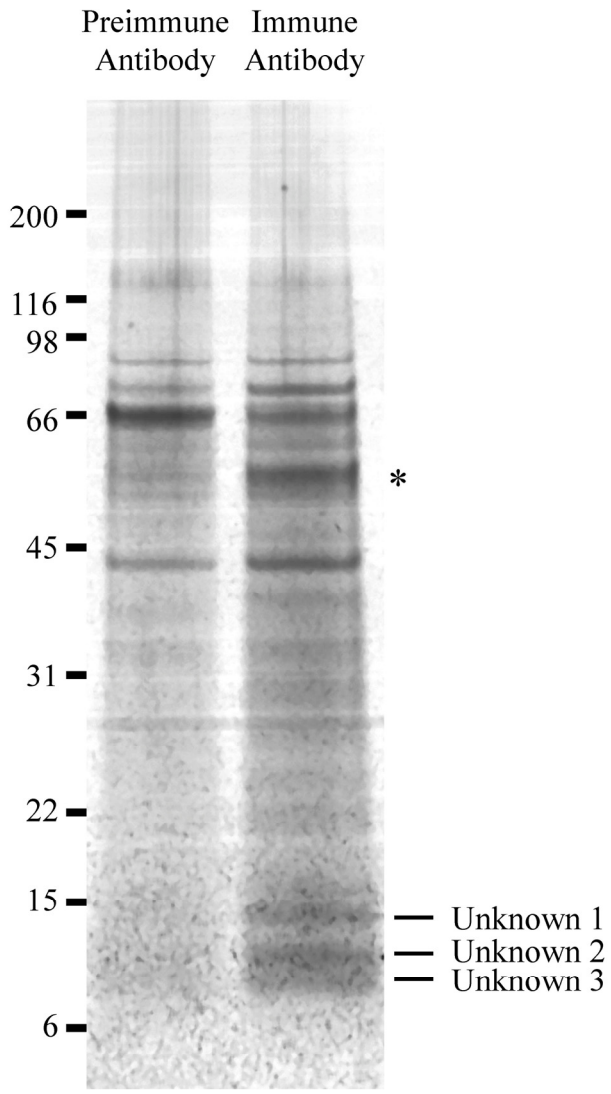

Figure 5. Immunoprecipitation using AtSNX2b antibodies. Coomassie blue-stained gel showing specific bands from immune antibody precipitation (right lane) and preimmune antibody precipitation (left lane). Immunoprecipitation of AtSNX2b protein complexes yielded three specific proteins not precipitated by preimmune antibodies. The proteins are named as Unknown 1 through 3. The asterisk shows a strong band in immune and a similar band is weakly present in the preimmune control.

CHAPTER 5. GENERAL CONCLUSIONS

Overview

In this dissertation I present results on the cell biology and genetics of Arabidopsis sorting nexin 2b (AtSNX2b). Sorting nexins are proteins involved in intracellular trafficking and are characterized by the presence of a PX domain. Sorting nexin proteins aid in targeting proteins to membranes, and initiate protein-protein and protein-lipid interactions. At the time this study was initiated, little information was available regarding plant sorting nexins and their functions. The past few years have yielded several publications showing the function of plant sorting nexins, specifically of Arabidopsis sorting nexin 1 (AtSNX1) and its homolog in Brassica (Vanoosthuyse et al., 2003; Jaillais et al., 2006). The functions of other plant sorting nexins remain elusive and this work aims to shed light on one of the previously uncharacterized sorting nexins in Arabidopsis, AtSNX2b. In this work, chapter 1 provided an overview of previous research on the sorting nexin protein family. In Chapters 2 and 3, I investigated the intracellular and *in planta* functions of AtSNX2b, showing AtSNX2b to be localized to endosomes and involved in endosomal trafficking and to have an effect on plant root growth. In Chapter 4 I investigated proteins that associate with AtSNX2b. Several proteins that interact with AtSNX2b were identified and other potential interactors discovered.

Cellular AtSNX2b study. Chapter 2 focused on the functional characterization of Arabidopsis AtSNX2b protein. I provided evidence that AtSNX2b is a functional sorting nexin. AtSNX2b protein has affinity for phosphatidylinositol 3-phosphate-rich membranes, allowing it to potentially cycle between endosomal structures. Localization experiments

suggest AtSNX2b is associated with the TGN and endosomes. AtSNX2b structures can be labeled with the endocytic tracer, FM4-64, and probably are involved in endocytosis. The overexpression of AtSNX2b produces enlarged endosomes and as a result inhibits N-terminal propeptide-specific trafficking towards the vacuole. The production of enlarged endosomes and possible inhibition of transport suggests that the role of AtSNX2b is likely in the mechanism of exit from endosomes. This chapter presents AtSNX2b as a sorting nexin functioning in protein trafficking and residing in endosomes.

***In planta* AtSNX2b study.** In chapter 3, *Atsnx2b* mutant analysis suggests involvement of the sorting nexin in responses to stress generated from limiting nitrogen, carbon or their combinations. Overexpression of AtSNX2b in plants results in an increase in root growth over that of wild-type. Growth of *Atsnx2b*-knockout (KO) roots is hindered compared to wild-type in both carbon/sucrose excess and limited situations and when nitrogen is limiting. This dual phenotype in carbon excess and limiting situations suggests that AtSNX2b possibly affects or is affected by the carbon/sucrose availability. Because carbon and nitrogen metabolism are interrelated (Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001), it is possible that AtSNX2b is involved in root growth as a positive growth regulator or as an indirect sensing and regulation system based on carbon and/or nitrogen available to plant cells. The nutrient stress and lack of AtSNX2b protein in null mutants potentially results in a combination of stresses that leads to slow root growth, while overexpression of AtSNX2b (AtSNX2b OX) can result in a stimulation of root growth. The chapter shows that early plant root growth processes involve AtSNX2b.

AtSNX2b-interacting factor study. The chapter 4 study attempts to analyze components of the AtSNX2b complex. Evidence of AtSNX2b localization to endosomal compartments was supported by colocalization of AtSNX2b in endosomal compartments with AtSNX1 and BRI1 (Russinova et al., 2004; Jaillais et al., 2006; Geldner et al., 2007; Jaillais et al., 2008). Evidence of BRI1 association with AtSNX2b was shown by detection of AtSNX2b in BRI1-GFP immunoprecipitations. Finally, demonstration of AtSNX2b association with the TGN-localized t-SNARE T7-SYP42 was obtained by detection of AtSNX2b in T7-SYP42 immunoprecipitations. Immunoprecipitation using AtSNX2b antibodies identified three additional proteins that potentially interact specifically with AtSNX2b. These proteins are not predicted to be related to known trafficking machineries. Further examination of their association with AtSNX2b should address their role in AtSNX2b-mediated trafficking.

Impact of Dissertation Study

All three studies presented in my dissertation have contributed new knowledge in understanding the function of Arabidopsis sorting nexin 2b. I provide evidence that a sorting nexin, AtSNX2b, is able to affect root growth in seedlings and this sorting nexin associates with BRI1 receptor kinase which is involved in growth and development. These data suggest that a trafficking protein can regulate growth and development. The understanding of protein trafficking in plants may eventually allow the improvement of agricultural crops by increasing yield and resistance to biotic and abiotic stress, as trafficking components can affect plant defense and stress adaptation (Carter et al., 2004; Lee et al., 2006; Sutter et al., 2006; Kalde et al., 2007; Robatzek, 2007). This study presents the role of a sorting nexin whose function can potentially affect endosomal transport. Depending on the stress imposed,

plant defense or stress adaptation may involve vesicle transport, endocytosis and exocytosis. Any effect on endosomal trafficking probably influences endocytosis and/or exocytosis and subsequently changes downstream signaling processes. Knowledge gained on the influence of endosomal trafficking on plant defense and adaptation will potentially allow the improvement of crop responses/adaptation to biotic or abiotic stress.

Future directions

This study characterizes AtSNX2b, showing intracellular functions in endosomes and trafficking towards the vacuole while its physiological functions reside in early root growth affected by nitrogen and carbon. In normal conditions, *Atsnx2b* KO plants show subtle differences in growth compared to wild-type plants. The function of AtSNX2b may be redundant with AtSNX2a due to high amino acid similarity (86% similarity) between the two proteins, and this could account for the subtle growth phenotype. Possibly, the AtSNX2a and AtSNX2b proteins separately or together will affect endosomal trafficking in early seedlings.

Growth of a seedling can be divided into several phases. First, seeds germinate and use seed storage proteins and lipids to grow and develop primary organs. Second, seedlings switch from using seed stores to photosynthesis and obtaining nutrients from the soil. Lastly, rapid development takes place to form roots and leaves to increase growth. During these phases the availability of nitrogen for amino acid synthesis and sugar for an energy source are important. Carbon and nitrogen are two of the most important elements essential for normal growth and metabolism (Kang and Turano, 2003). Appropriate carbon:nitrogen ratios in the cell are maintained by transporters or other mechanisms which take up sugars, nitrite, nitrate and ammonium into the cell.

From this study, a model for AtSNX2b function has been developed. [1] AtSNX2b is involved in endosomal trafficking and recycling; and [2] knocking out AtSNX2b leads to potentially a significant decrease in transport and/or recycling in endosomes. This decrease slows the rate of endosomal transport in recycling endosomes, from PM-to-endosome and/or between early and late endosomes. The function of AtSNX2b may involve three aspects which could occur separately or in combination to cause the physiological effects seen in *Atsnx2b* KO plants. First, localization of sucrose and nitrogen transporters to the PM; second, endocytosis of sugars and organic molecules; and lastly, the localization of PM receptors that affect growth.

The uptake of sugars and nitrogen occur done through sucrose and nitrogen transporters at the PM or though a recently-proposed bulk uptake in plants via endocytosis (Etxeberria et al., 2005; Paungfoo-Lonhienne et al., 2008). The PM transporters take in specific sucrose or nitrogen derivatives for usage, while endocytosis takes in sugar and organic complexes to the vacuole for breakdown into simpler derivatives which are readily utilizable by the cell. The aspects of AtSNX2b function in KO plants may lead to: [1] Fewer sugar or nitrogen transporters at the PM would decrease overall uptake of sugars and nitrogen derivatives required by the cell for growth. [2] A decrease in endocytic trafficking of endocytosed sugar and organic complexes to the vacuole for breakdown into usable derivatives would lessen also the overall availability of sugar and nitrogen derivatives. Because the carbon and nitrogen availability inside the cell contributes greatly to growth in seedlings, a reduction would result in reduced growth rate in general. [3] Since many PM proteins cycle constitutively through endosomes, slowed endocytic trafficking could potentially reduce the number of PM proteins that regulate growth and slow their

internalization or recycling. Even if PM receptors such as auxin-related or brassinosteriod-related receptors still continue to signal to promote growth, the bottleneck of insufficient amino acids or energy would inhibit growth. The effect is an overall reduction in growth rate observed. In the case where KO plants are subjected to no nitrogen or no sucrose, the stress of altering carbon and nitrogen availability in addition to slowed transport in KO plants could contribute to the reduced root growth. The model can be partially supported by the observation that older *Atsnx2b* KO plants are observed to have increases in secondary and tertiary root development when compared to wild-type plants (unpublished). This can be seen as an adaptive mechanism to increase root surface area for uptake in response to the decreased transport ability in the whole plant. In addition, further support for the model is that plants overexpressing AtSNX2b are observed to have a faster root growth rate than wild-type plants.

To test this model, several questions have to be addressed: [1] Does AtSNX2b have redundant functions with AtSNX2a, being highly similar? If so, will a double KO for AtSNX2a/2b more severely affect endosomal trafficking and/or recycling?; [2] Is there a lower number of sugar or nitrogen transporters at the PM compared to the WT?; [3] Is there a decreased rate of transport of new PM proteins or a decreased rate of internalization/recycling at the PM?; [4] Is there a slower rate of endocytosis at the PM compared to wild-type?; [5] Is there decrease rate of endosomal recycling from and to late endosomes?; [6] lastly, in overexpression lines of AtSNX2b, is endosomal trafficking enhanced? The studies in this dissertation have laid the groundwork for further investigation of sorting nexins as follows:

Analysis of various AtSNX2b mutants. Studies in this dissertation have shown a probable role for AtSNX2b in endosomes and a probable plant function of AtSNX2b affecting root growth; in addition, a model of the role of AtSNX2b in plants was proposed above. To address the validity of this model, several KOs have to be generated and analyzed. The following studies will address the questions presented above. First, analysis of *Atsnx2a* and *Atsnx2b* single and double mutants in endosomal trafficking as well as in carbon and/or nitrogen stress will address the question of redundancy. Second, a comparison of [i] the relative number of sugar or nitrogen transporters and growth-related receptors (like PINs and BRI1) by quantitative enzyme-link immunosorbent assay (ELISA) and [ii] the relative PM transporter/receptor distributions at the PM versus in endosomal locations in the KO mutants to WT, or AtSNX2b OX to WT will address if a reduction in PM transporters can lead to reduced seedling root growth. Third, a comparison of the recycling of PM proteins such as PIN-FORMED (PIN) proteins or PM syntaxins SYP121, SYP122 and SYP123 in WT compared to KO mutants or OX will address the question of a decreased rate of PM recycling by endosomes. Fourth, slowed endocytosis from the PM towards late endosomes and to the vacuole can be tested by examining effects of single and double KO mutants on the endocytosis of FM4-64 tracer and the vacuolar targeting of N-terminal propeptide (NTPP)-containing proteins. Fifth, slowed endocytic trafficking/recycling from late endosomes to Golgi can be address by comparing the localizations retromer components VPS29, VPS35 or VPS26 in the KO mutants. And lastly, the AtSNX2b OX will be examined for an effect on endocytosis and trafficking to the vacuole of NTPP-containing proteins; this addresses whether overexpression can enhance endosomal trafficking. Phenotypic comparisons among mutants will provide more evidence and confirm the probable role of each protein in root

growth and possibly plant development. Additionally, by analyzing single and double mutants the relative contributions of each sorting nexin trafficking, root growth and potentially plant development will be addressed. This series of experiments addresses the validity of the model proposed above for the role of AtSNX2b.

Investigation of AtSNX2b in internalization and trafficking. Plant growth and development depends on detection and response to environmental signals such as hormones and other ligands (Xiao et al., 2000; Li et al., 2002; Nam and Li, 2002; Gifford et al., 2005; Chen et al., 2006; Huang et al., 2006). In this dissertation we have shown a potential interaction of AtSNX2b with BRI1-GFP by immunoprecipitation and colocalization. Receptor-mediated endocytosis of BRI1 is increased after binding to the coreceptor BAK1 as a result of brassinosteriod binding (Russinova et al., 2004) to generate a type of endosomal signaling compartment for plant receptor-kinase pathways (Geldner et al., 2007). From the model proposed for the role of AtSNX2b, the role of AtSNX2b in BRI1 signaling endosomes establishes a potential specific function for AtSNX2b. AtSNX2b can potentially function in a general endocytic process and/or in specific internalization steps. To tests the specificity of AtSNX2b in the internalization process, the analysis of endocytosis of AtSNX2b with various endocytic markers should be done. Well-studied markers such as BRI1-GFP, PIN1-GFP, PIN2-GFP and BOR1-GFP will be tested. BOR1-GFP is a boron transporter on the PM internalized by endocytosis at high boron concentrations (Takano et al., 2005). BOR1-GFP can function as a ligand-inducible endocytic marker similar to BRI1-GFP. PIN1-GFP and PIN2-GFP are efflux carriers for auxin which cycle through endosomes (Blilou et al., 2005; Xu and Scheres, 2005) and represent constitutive endocytosis. Several lines with these markers need to be made: [1] fluorescent-tagged AtSNX2b with these markers; and [2]

expression of these markers in the *Atsnx2b* KO and *AtSNX2a/2b* double KO background. Information from the comparison among the endocytic markers will establish the function of AtSNX2b in a general endocytic process or also in specific internalization steps.

The detailed examination of single and double KOs in endocytosis of well-studied endocytic markers (mentioned above) will provide specific roles and individual contributions of each sorting nexin or both together in the internalization process. The potential function of sorting nexins in signaling may be established from association with BRI1. Reduction, inhibition or an increase of trafficking of these endocytic markers may suggest an alteration of trafficking of components involved in growth and development.

Investigation of unknown interacting factors of AtSNX2b. A study in this dissertation identified three potential interacting factors with AtSNX2b immunoprecipitations. These factors are not predicted to be involved in protein trafficking. Their localizations are predicted to range from PM, to the mitochondria and to the nucleus. PM-related proteins are the most likely candidates to function with endosome-located AtSNX2b. It is uncertain how the mitochondrial and nuclear located proteins can interact with AtSNX2b; possibly the proteins are not truly associated with AtSNX2b complex or their predicted localization is wrong. The localization of these factors and colocalization with AtSNX2b needs to be tested. Fluorescent tagged proteins will be used to determine their intracellular localization and colocalization with AtSNX2b, and tagged protein will be used for *in vitro* expression and pull down assays to test for association with AtSNX2b. In addition, the expression and localization of these proteins in *Atsnx2b* KO, *Atsnx2a/2b* double KO or overexpression plants will be tested to determine whether the AtSNX2b affects the distribution, location and stability of the unknown proteins.

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APPENDIX. A SYSTEM FOR PRODUCTION OF PHARMACEUTICAL PROTEINS BY ACCUMULATION IN PROTEIN BODIES OF SOYBEAN SEEDS

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Abstract

Molecular pharming in plants provides a cheap and scalable system for production of pharmaceutical proteins. Plant seeds provide an environment to accumulate these proteins but they are subjected to degradation by vacuolar proteases when the protein is targeted to the protein storage vacuole. By targeting recombinant proteins to soybean protein bodies away from proteases in the vacuole, recombinant proteins can accumulate to high quantities without risking degradation. We provide here a potential method to accumulate high levels of foreign proteins in protein bodies in soybean seeds to avoid degradation.

Introduction

Over the past decades, plant biotechnology has revolutionized the production and manufacturing industries (Twyman et al., 2003). Pharmaceutical proteins from natural sources have the potential for being contaminated and are expensive to purify (Fischer and Emans, 2000; Ma et al., 2003). These proteins can be produced cheaper, faster and are easier to purify from heterologous systems (Fischer and Emans, 2000; Giddings, 2001). Several systems can be used for expression of these proteins (Giuliani, 2007) and while each has their

own advantages and disadvantages, plants provide a safe, inexpensive and almost limitless supply while requiring minimal effort (Ma et al., 2003). This molecular farming system in plants is a safe and scalable system for large-scale protein synthesis and has been increasing in popularity (Fischer and Emans, 2000; Giddings, 2001).

In the plant-based system for production of pharmaceuticals, seeds have the advantage of accumulating proteins in a small volume and stable environment (Stoger et al., 2005). Soybean seeds provide an ideal environment/system for producing large amounts of protein. Soybean is an agriculturally important crop with protein-rich seeds containing up to 51% protein and 25% oil per dry weight of seed (Huskey et al., 1990). Soybean seeds contain two major proteins, 11S glycinin and 7S β-conglycinin, that accumulate in the protein storage vacuole (PSV) (Horisberger et al., 1986). These proteins accumulate during seed development and are mobilized to provide nutrients during germination and early seedling development. Proteins that are destined for the PSV are translocated into the endoplasmic reticulum (ER), travel to the Golgi and are further transported in dense vesicles (DV) which fuse with the PSV (Jolliffe et al., 2005). Of the major proteins in soybean, the trafficking of β-conglycinin requires a C-terminal vacuolar sorting signal while glycinin does not possess a typical vacuolar sorting signal, but requires β-conglycinin to reach the PSV (Mori et al., 2004).

Use of the β-conglycinin targeting signal would hypothetically allow the accumulation of foreign proteins in the PSV. However, accumulation of foreign proteins in soybean PSVs has failed due to degradation by vacuolar proteases (Philip et al., 2001). This problem can be overcome by separating the proteins being produced from the vacuolar proteases, allowing high accumulation while preventing degradation.

Kinney et al. (2001) have shown that silencing expression of the α' and α subunits of β-conglycinin increases accumulation of glycinin in endoplasmic reticulum (ER)-derived protein bodies. The authors also show that these protein bodies do not contain markers for the PSV, indicating that it is a non-vacuolar compartment. Since these protein bodies are a non-vacuolar compartment and are also present in wild-type seeds, they are an ideal location to target proteins to avoid degradation by vacuolar enzymes. In this study, we take advantage of the glycinin trafficking system to protein bodies. Green fluorescence protein (GFP)-fused glycinin constructs were made and the localization of the fusion proteins were examined in soybean. Localization of GFP fusions in soybean leaves and seeds indicates that the fusion accumulates in structures that do not resemble the PSVs in both seeds and leaves. We hypothesize that these are protein bodies but further studies are needed to verify the identity of the GFP-labeled structures.

Materials and Methods

Plasmid Construction. Glycinin was amplified from soybean seed cDNA using primers 5'-CCATGGCCAAGCTTGTTCTTTCCCTTTGTTT-3 (forward; Gy2-forward) and 5'-AAGCTTAGCCACAGCTCTCCTCTGAG-3' (reverse). GFP was amplified from pJ4-GFP-XB vector (Igarashi et al., 2001) using primers 5'-AAGCTTATGGTGAGCAAGGGCGAGGA-3' (forward) and 5'-TCTAGATTACTTGTACAGCTCGTCCATGCCG-3' (reverse; GFP-reverse). A two step PCR extension was used to join and amplify Glycinin and GFP using end primers Gy2-forward and GFP-reverse to yield Glycinin-GFP. The Glycinin-GFP encoding fragment was digested with *Nco*I and *Xba*I and subcloned into *Nco*I-*Xba*I digested pRTL2 vector

(Carrington and Freed, 1990) to generate pRTL2::35S-Glycinin-GFP. Restriction sites are underlined.

To generate Glycinin-GFP for expression from the glycinin promoter (Glypro), glycinin promoter was amplified from soybean seed genomic DNA using primers 5'-CTGCAGTTAATTGATTGATATGTCATTACT -3' (forward) and 5'-GAATTCGTGATGAGTGTTCAAAGACAATGG-3' (reverse). This was followed by subcloning of *Pst*I-*Eco*RI digested Glycinin promoter into pRTL2 digested with *Pst*I-*Eco*RI. This generated pRTL2::Glypro-Glycinin-GFP. Restriction sites are underlined.

The terminator for vegetative storage protein (T_{vsp}) was amplified using 5'-GAGCTCTATTGCACTCCCTTTTAACT-3' (forward) and 5'-GAATTCCCGGACGGGGCGGTACCGGC-3' (reverse) from pTF101.1. T_{vsp} was digested with *Sac*I and *Eco*RI and subcloned into *Sac*I-*Eco*RI digested pTF101. Restriction sites are underlined.

Final assembly was done with pTF101 vector (kindly provided by Dr. Kan Wang). GlyPro-Glycinin-GFP and 35S-Glycinin-GFP fragments from pRTL2 were ligated into pTF101. Each construct was digested with *Pst*I and *Xba*I and subcloned into pTF101 vector digested with *Pst*I and *Xba*I to generate pTF101::GlyPro-Glycinin-GFP-T_{vsp} and pTF101::35S-Glycinin-GFP-T_{vsp} plasmids. A scheme for the construction is outlined in Figure 1.

Bombardment of Soybean Seeds and Leaf. Soybean *Glycine max* cv Thorne seeds and leaves were used in bombardment procedures. The first trifoliate leaves were used in the bombardment of pTF101::35S-Glycinin-GFP-T_{vsp}, while developing seeds 2 weeks prior to

desiccation were used in bombarding pTF101::Glypro-Glycinin-GFP-T_{vsp}. Gold particles (0.6μm) were coated with plasmid DNA of interested following protocols from the Plant Transformation Facility (Iowa State University; http://www.agron.iastate.edu/ptf/protocol/Gold%20Particle.pdf). Plasmid DNA (30μg per shot) was delivered into seeds or leaves through a metal micron mesh at 900psi at 12cm distance.

Samples were then incubated in the dark for 24 h to allow sufficient expression for visualization. Fluorescent signal detection and documentation was performed using a confocal laser scanning microscope (Leica TCS/NT, Leica Microsystems, Exton, PA, USA). The confocal laser microscope utilizes a Krypton 568nm and Argon 488nm laser for excitation. Filters for emission were RST588 BP525±25 (FITC-specific detection) and LP590 (TRITC-specific detection). FITC was used to check GFP expression, while TRITC was used to verify cell boundaries when needed. Images were further processed for graphic presentation using Adobe Photoshop (Adobe Systems, Mountain View, CA, USA) and MetaMorph (Molecular Devices, Sunnyvale, CA, USA).

Results

Plasmid Construction. To examine the possibility of targeting proteins to protein bodies in soybean seeds, glycinin was tagged with GFP, GFP expression was confirmed, and the possibility of expression in seeds was analyzed. To do this, two promoters were used for expression of the GFP-tagged glycinin construct – a constitutive 35S promoter and a seed specific glycinin promoter. Since the orientation of GFP at the N-terminus or C-terminus of the glycinin protein may have an effect on the targeting into protein bodies, fusions with GFP

at either end of the glycinin protein will be made and tested. The protein fusions formed will be GFP-glycinin and glycinin-GFP. For targeting to the protein bodies, the fusions require an N-terminal ER signal sequence. Since glycinin is a seed storage protein that is located to ER bodies, it already contains a targeting sequence and does not require the addition of an extra targeting signal. GFP-glycinin on the other hand does not have a targeting signal sequence at the N-terminus of GFP. An ER signal sequence from glycinin must be added in front of GFP to target GFP-glycinin properly into the ER as a prerequisite for targeting to protein bodies.

Constructs for glycinin-GFP under both the 35S promoter and glycinin promoter were finished. A diagram of the cloning scheme for glycinin-GFP is shown in Figure 1. The glycinin-GFP fragments were finally cloned into the pTF101 vector.

Expression of Glycinin-GFP. To verify that the glycinin-GFP construct is functional, 35S::glycinin-GFP was bombarded into soybean leaf and expression of GFP was analyzed. Expression of 35S::glycinin-GFP can be seen 24 h after bombardment (Figure 2; arrows). A small amount of GFP can be seen in discrete structures while GFP staining can also be seen diffuse in the cytoplasm. This suggests that glycinin-GFP can be targeted to some non-vacuolar structures in leaves.

To further determine the localization of glycinin-GFP, Glypro::Glycinin-GFP was used to bombard soybean seeds. As a control, GFP alone was used to bombard seeds to determine whether punctuate structures seen were specific. GFP expression alone in seeds shows that GFP is diffuse throughout different cells in the seed (Figure 3B). Glycinin-GFP expressed in soybean seeds accumulated in punctuate structures (Figure 3A). An enlarged image of bombarded seed shows that these structures do not appear to be vacuoles. The structures are distinct and more in numerous than those found in leaves. This suggests that

the GFP structures generated are specific for glycinin-GFP, are not vacuolar, and may be therefore protein bodies.

Discussion

In soybeans, identification of the presence of ER-derived protein bodies was the result of reduced β-conglycinin levels leading to accumulation of glycinin in a non-vacuolar structure (Kinney et al., 2001). This study uses this idea to target foreign proteins to protein bodies, which are distinct from the PSV. Protein bodies do not possess degradative enzymes present in the PSV, and this allows the development of a production system for molecular farming. Here we are able to show that in bombardment of leaves with the fusion protein glycinin-GFP, GFP-tagged protein accumulates in certain small structures that do not resemble a PSV. GFP alone produces a diffuse pattern of staining in seeds, whereas glycinin-GFP in seeds forms one to several structures per cell accumulating GFP. Judging from the size and number in leaf cells, the structures are hypothesized to be protein bodies. The GFP structures are small and spherical, and take up only a small fraction of a whole cell. Both bombardment in leaf and seeds show that the structures are consistent in size and number and do not appear vacuolar.

We present here possible evidence that the glycinin-GFP expression system in soybean leaf and seeds localizes glycinin-GFP to structures that resemble ER-derived protein bodies and not PSVs. However, further experiments are required to address whether these structures are actually ER-derived protein bodies, such as co-localization with ER and vacuolar markers.

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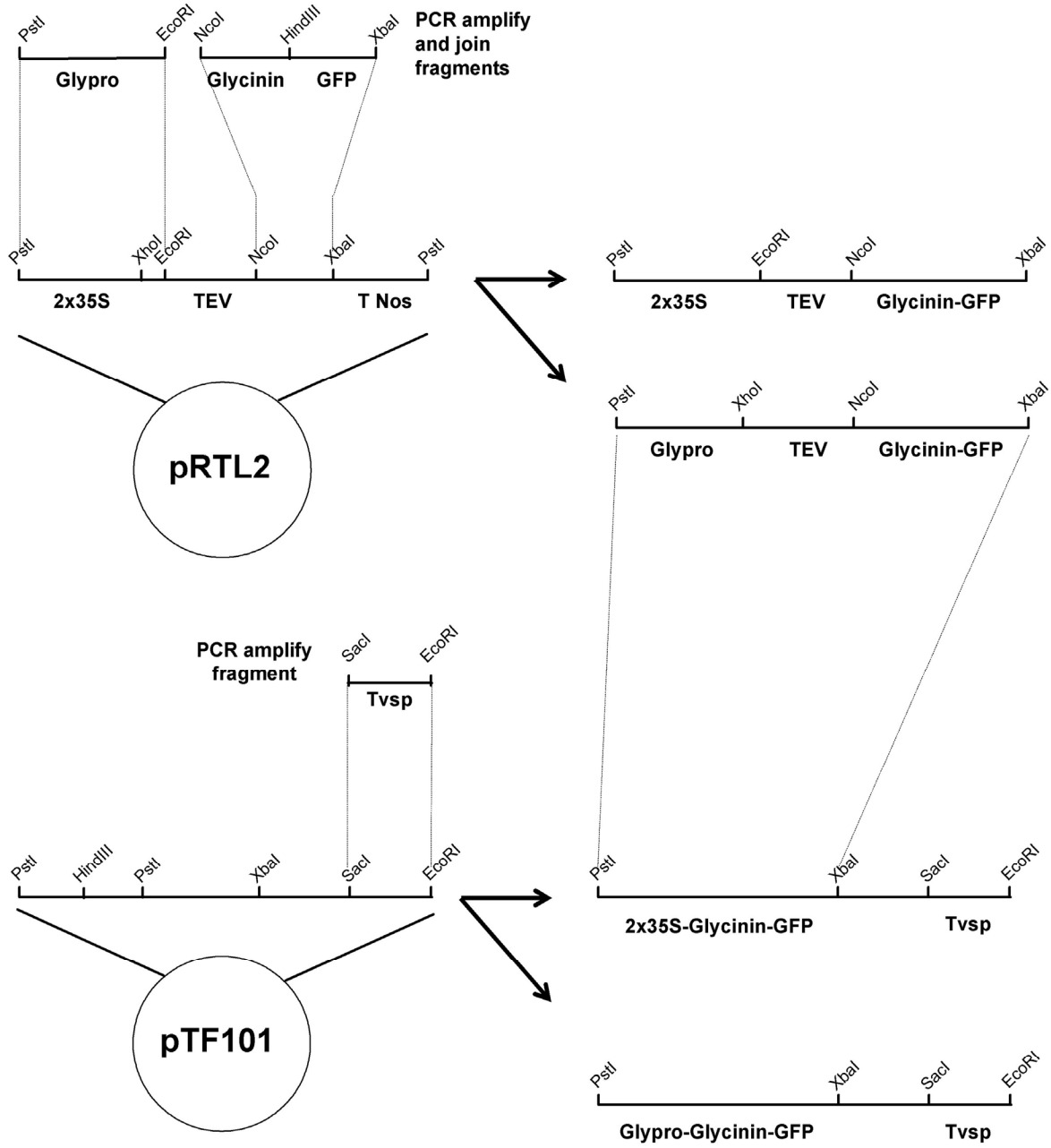

Figure 1. The cloning scheme for Glycinin-GFP. Glycinin-GFP was cloned behind a 35S constitutive promoter and a seed-specific glycinin promoter (Glypro). Fragments were cloned into pRTL2 and the entire cloning cassette from pRTL2 was subcloned into pTF101 to generate pTF101::GlyPro-Glycinin-GFP-T_{vsp} and pTF101::35S-Glycinin-GFP-T_{vsp} plasmids.

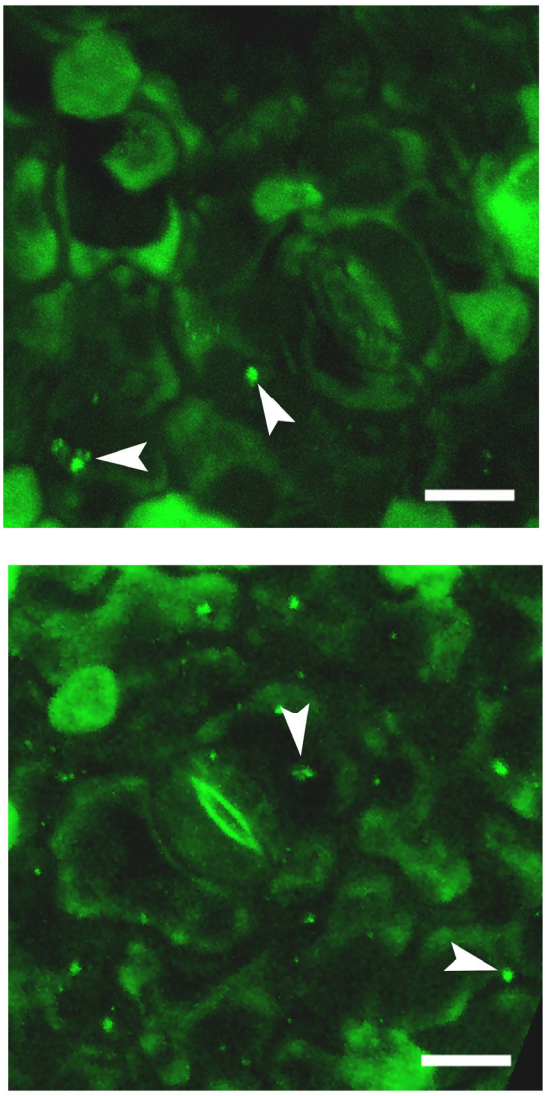

Figure 2. Soybean leaf bombardments with 35S-Glycinin-GFP. 30µg of plasmid were bombarded into leaves and allowed to express for 24 h. Pictures show two independent bombardments on soybean leaves. Arrows show GFP accumulation in small structures (arrows). Scale bars are 20µm.

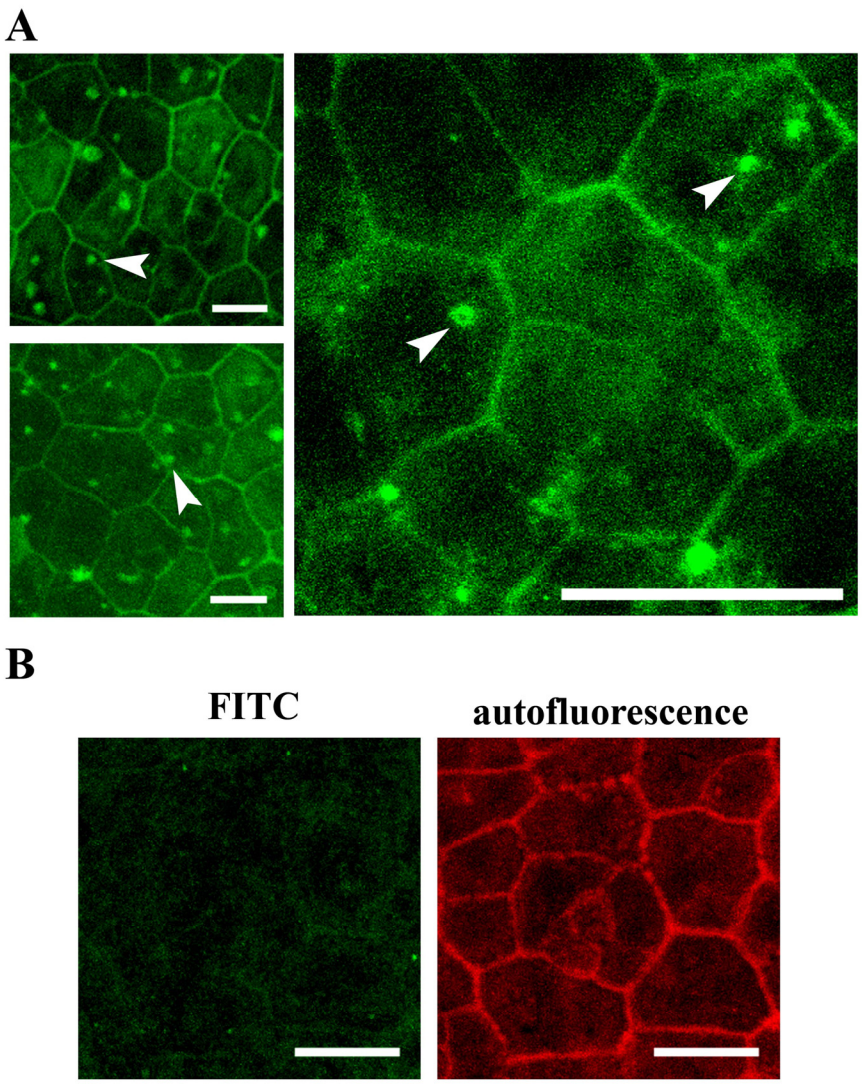

Figure 3. Soybean seed bombardments with Glypro-Glycinin-GFP. 30µg of plasmid were bombarded into developing seeds and allowed to express for 24 h. A, Glypro-Glycinin-GFP was delivered into developing seeds. *Left*, two independent bombardments showing GFP structures. *Right*, enlarged picture of bombarded seed cells. The images show GFP accumulating in small structures (arrows). B, a GFP control plasmid bombarded into seeds shows a dispersed pattern of GFP in cells. Auto-fluorescence from seeds shows cell boundaries. No GFP-labeled structures can be seen inside a cell. Scale bars are 20µm.